

**STUDIES ON THE METABOLISM OF
PHENYLPROPANOIDS AND RELATED COMPOUNDS
IN *CAPSICUM FRUTESCENS***

by

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**A THESIS PRESENTED IN FULFILLMENT
OF REQUIREMENTS FOR THE DEGREE OF
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DECLARATION

**I hereby declare that this thesis was
composed by myself, and the work described
herein to be my own, except where indicated
otherwise.**

[Redacted Signature]

Sukrasno

Edinburgh, 1991

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ABBREVIATIONS

A/abs	absorbance
AlCl ₃	aluminium chloride
BSA	bovine serum albumine
BuOH	n-butanol
c	approximately (<i>circa</i>)
°C	degrees centigrade
C ₆ H ₆	benzene
CHCl ₃	chloroform
Ci	currie
cm	centimetre
CPA	p-chlorophenoxyacetate
CPM	counts <i>per</i> minute
cv.	cultivar
d.	day(s)
2,4-D	2,4-dichlorophenoxyacetate
DPM	disintegrations <i>per</i> minute
DW	dry weight
ed(s).	editor(s)
<i>et al.</i>	<i>et alia</i>
Et ₂ O	diethylether
EtOAc	ethylacetate
EtOH	ethanol
Fr.	fraction
FW	fresh weight
g	gram
xg	x gravitational force
h.	hour(s)
HCl	hydrochloric acid
H ₂ SO ₄	sulphuric acid
HPLC	high performace liquid chromatography
i.e.	that is
l	litre
LLE	liquid-liquid extraction
LSC	liquid scintillation counting (counter)
λ	wavelength
M	molar
m	metre
mCi	millicurrie
mg	milligram
max	maximum
min.	minute
ml	millilitre
mM	millimolar
mm	millimetre
mol.	mole
MS	Murashige and Skoog
MW	molecular weight
N	normal
NaHCO ₃	sodium bicarbonate
NaOAc	sodium acetate

NaOMe	sodium methoxide
nm	nanometre
NMR	nuclear magnetic resonance
OD	optical density
PAL	phenylalanine ammonia-lyase
PBD	2-phenyl,5-biphenyl-oxadiazol
PFP	p-fluorophenylalanine
POPOP	1,4-di-2-(5-phenyloxadiazolyl)-benzene
PPO	2,5-diphenyloxazol
PVP	polyvinylpyrrolidone
r	coefficient correlation
rel.	relative
Rf	retention factor
RITA	Radioactivity Intelligent Thin layer Analyser
rpm	revolutions/minute
Rt	retention time
SA	specific activity
SE	standard error of the mean
sec.	second
SH	Schenk and Hildebrandt
TCA	trichloroacetate
TLC	thin layer chromatography
2D-TLC	two dimensional TLC
μkat	microkatal
μCi	microcurie
μm	micrometre
μl	microlitre
μmol	micromole
UV	ultraviolet
v/v	volume <i>per</i> volume
Var.	variety
w/v	weight <i>per</i> volume

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ABSTRACT

The aim of this project was to investigate the relationship between the metabolism of various secondary metabolites derived from cinnamic acid (phenylpropanoids and related compounds) and the synthesis of capsaicin in fruits and cell cultures of *Capsicum frutescens*.

A variety of compounds derived from cinnamic acid ^{were} was detected in chilli pepper cultures. However, free phenolics were not detected, in callus cultures. Most of the conjugated phenolics were present as O- or ester glycosides. p-Coumaric, ferulic and vanillic acid were detected after release by alkaline, acid or enzymic hydrolysis of water soluble conjugated phenolics. In suspension cultures, several unknown free phenolics were detected in the culture medium. Again as with callus, most phenolics in the cells were present as water soluble conjugates. Over the growth cycle, both callus and suspension cultures accumulated a substantial amount of lignin, but capsaicin was not detected.

Apart from capsaicin, a number of water soluble conjugated phenolics were also accumulated in chilli pepper fruits. p-Coumaroyl, caffeoyl and 3,4-dimethoxycinnamoyl glycosides (hydroxycinnamoyl glycosides); vanillic acid glycoside, 3-rhamnosylquercetin, 7-glucosylluteolin and two unknown flavonoid glycosides were all detected. In addition, several unknown water soluble conjugated phenolics were also present. The proposed free phenolic intermediates in capsaicin biosynthesis were present at vanishingly low levels and only detected after short pulse labelling with [U-¹⁴C]-cinnamic acid. The synthesis, accumulation and disappearance of these compounds was studied in relation to the onset of capsaicin synthesis.

Before the onset of capsaicin synthesis, the main products of phenylpropanoid metabolism were the three hydroxycinnamoyl glycosides. During the onset of capsaicin synthesis, fruits were also actively synthesizing lignin and C₆-C₁ phenolic glycosides. There are two major sources of the intermediates for capsaicin, lignin and

C₆-C₁ phenolic glycosides, 1) the hydroxycinnamoyl glycosides and 2) free phenolics directly synthesized from cinnamic acid. After the onset of capsaicin synthesis, fruits were still actively metabolizing cinnamic acid to produce C₆-C₁ phenolic glycosides. Some of these glycosides appeared to be further metabolized during fruit ripening.

The metabolism of phenylpropanoids in developing fruits and cell cultures is discussed in relation to the regulation of capsaicin synthesis.

Chapter 1: INTRODUCTION

1. INTRODUCTION

Secondary metabolites and their uses

Secondary metabolites can be defined as compounds with no recognized role in the maintenance of fundamental life processes in the organisms in which they occur (Bell, 1981). Plants differ from animals in their capacity to produce secondary metabolites and this could be related to differences in mobility and the ability to remove unwanted materials. Mankind has been exploiting the secondary metabolites produced by plants, especially for medicines, flavours and perfumes, for thousands of years, despite a lack of understanding of how these compounds are used by the producing plants. Indeed, until the beginning of the twentieth century almost all medicines were obtained from plants (Fowler *et al.*, 1990). Recently Farnsworth (1990) has shown that 80% of people living in the developing countries still rely almost completely on medicines extracted from plants. His survey between 1959 and 1980 also revealed that 25% of all prescriptions dispensed in the USA contained active principles derived from plants. These observations show the importance of plant secondary metabolites to man.

Capsaicin, the main pungent principle of chilli pepper fruits is one of many secondary metabolites extracted from plants which are used commercially. Acetone extracts of chilli pepper fruits (*capsicum oleoresin*) which contain capsaicin and its analogues (capsaicinoids) are used as a component of counter irritants and also as a carminative (Reynolds and Prasad, 1982). However, chilli pepper is perhaps best known as a spice that can, in moderate amounts, stimulate appetite, but at high concentration may reduce the digestibility of the food by increasing gastric motility. Since capsaicin is commercially valuable, there have been attempts with limited success to produce it using plant cell cultures (Yeoman, *et al.*, 1980; Lindsey, 1985, 1986a; Lindsey and Yeoman, 1983, 1984a,b). However, in order to increase capsaicin yield consistently, it is essential to gain a better understanding of the biosynthetic pathway the early part of which is shared with phenylpropanoid

metabolism in fruits and cultured cells (Hall *et al.*, 1986, 1987; Holden, *et al.*, 1987a; Yeoman *et al.*, 1989; Hall and Yeoman, 1991).

Accumulation of capsaicin in plants and cell cultures

Capsaicin is only found in the genus *Capsicum* of which there are 20 to 30 wild and three to four domesticated species (McLeod *et al.*, 1979). It has also been suggested that the presence of capsaicin may be used as a taxonomic character for certain species of the genus *Capsicum* (Eshbaugh, 1977). The four domesticated species of *Capsicum* according to Pickersgill *et al.* (1979) are *Capsicum baccatum*, *Capsicum annuum*, *Capsicum chinense* and *Capsicum frutescens*. There is a great variation in fruit size, shape and colour as well as pungency even within one species. Non pungent cultivars can be found in *C. annuum*, *chinense* and *baccatum*, but not in *C. frutescens* (Smith, 1987). Indeed, the fruits of *C. frutescens* var. cayenne used in this study are highly pungent.

In chilli pepper plants, capsaicin is only accumulated in fruits at one particular stage of development (Iwai *et al.*, 1979; Holden *et al.*, 1987a). Previously it was thought that capsaicin accumulation was related to fruit ripening as indicated by a colour change from green to red brought about by the active accumulation of carotenoids. But experimental evidence now suggests that there is no correlation between the levels of capsaicin and carotenoids in developing fruits (Suzuki *et al.*, 1980). It is known that the time from anthesis to the onset of capsaicin accumulation varies depending on the environmental conditions (Balbaa *et al.*, 1968), but this can be closely controlled when plants are grown under strict conditions. It has also been shown that a loose correlation also exists between the increase in fruit length and the onset of capsaicin accumulation (Holden *et al.*, 1987a; Yeoman *et al.*, 1989). Therefore, labelling fruits at anthesis can provide a means of generating a reasonably uniform population for experimentation (Holden *et al.*, 1987a, Holland, 1989).

In fruits, capsaicin is only accumulated in the epidermal cells of placental tissue (Balbaa *et al.*, 1968; Iwai *et al.*, 1979; Suzuki *et al.*, 1980, Rowland *et al.*, 1983). However, during handling the seeds and pericarps become contaminated with

capsaicin from the placenta (Rowland *et al.*, 1983, Holland, 1989). Five different capsaicinoids (capsaicin and its analogues) have been reported in chilli pepper fruits. These are capsaicin, homocapsaicin, dihydrocapsaicin, homodihydrocapsaicin and nordihydrocapsaicin. Structurally, they differ only in the presence or absence of the double bond at the 6 position on the side acyl chain or the length of the acyl moiety (9 to 11 carbon atoms). Capsaicin and dihydrocapsaicin are normally the major components (Bennet and Kirby, 1968; Suzuki *et al.*, 1981) and represent at least 95% of the total capsaicinoid content.

Attempts to produce capsaicin in plant tissue and cell cultures started in 1969 (Baier *et al.*, 1969). Although the callus cultures studied were derived from capsaicin producing tissue (placenta), capsaicin was not detected in the cells. However, this does not necessarily mean that capsaicin was not synthesized, because it was shown later that capsaicin is released almost completely into the medium from cell cultures (Yeoman *et al.*, 1980; Lindsey and Yeoman, 1983). A variety of empirical approaches have been used to increase capsaicin production in cell cultures. These include precursor feeding (Yeoman *et al.*, 1980; Lindsey, 1986a, Johnson *et al.*, 1990); cell immobilization (Lindsey, 1985, 1986a,b; Lindsey and Yeoman, 1983, 1984; Lindsey *et al.*, 1983; Ravishankar *et al.*, 1988; Johnson *et al.*, 1990), nutrient limitation (Lindsey, 1985, 1986b), cell line selection (Aitken and Yeoman, 1986; Salgado-Graciglia and Ochoa-Olejo, 1990) and elicitation (Holden *et al.*, 1988a,b). Despite these efforts, the amount of capsaicin accumulated in cell cultures is still lower than in fruits. Therefore, a better understanding of the regulation of capsaicin biosynthesis is required so that relevant strategies can be adopted to develop cultures which consistently yield a high level of capsaicin.

The biosynthesis of Capsaicinoids

There are two distinct arms of the pathway which lead to the formation of capsaicinoids. The aromatic moiety is derived from phenylalanine (Bennet and Kirby, 1968; Leete and Loudon, 1968), while the acyl component is derived from valine or leucine, and acetate (Leete and Loudon, 1968; Kopp *et al.*, 1980; Suzuki *et al.*, 1981).

Valine and leucine are precursors for fatty acids with even and odd numbers of carbons respectively. Studies on the intermediates of capsaicinoid biosynthesis have concentrated on the aromatic part of pathway, possibly due to the more complex nature of the reactions involved in the conversion of phenylalanine to the aromatic moiety of capsaicin and their association with general phenylpropanoid metabolism. Indeed, it has been proposed that the aromatic moiety is synthesized from phenylalanine via cinnamic acid and its hydroxy derivatives followed by chain shortening (C_6-C_3 to C_6-C_1) and transamination (Bennet and Kirby, 1968). Radiolabelling with the proposed intermediates or short pulse labelling with radiolabelled phenylalanine or cinnamic acid tend to support this pathway (Bennet and Kirby, 1968; Hall *et al.*, 1987; Holden *et al.*, 1987a; Yeoman *et al.*, 1989, Hall and Yeoman, 1991). Fig. 1.1. shows the proposed biosynthetic pathway from phenylalanine leading to capsaicin.

There is no doubt that the activities of the enzymes catalysing the conversion of phenylalanine to ferulic acid are present in fruits, because this is the core pathway in phenylpropanoid metabolism (Hanson and Havir, 1979). The presence of the enzyme responsible for the condensation of vanillylamine and the fatty acid component has been demonstrated in fruits by Iwai *et al.* (1977), Fujiwake *et al.* (1982) and Holland (1989). The enzymes which catalyse the conversion of ferulic acid to vanillin, and vanillin to vanillylamine have not been studied. Also there may be an alternative pathway for the formation of vanillin as proposed by Yeoman *et al.* (1980) in which vanillin is synthesized from caffeic acid via protocatechuic aldehyde (see Fig. 4.7).

It has been mentioned earlier that the pathway leading from phenylalanine to ferulic acid forms the core of reactions in phenylpropanoid metabolism, which means that the precursors for capsaicin are also destined to become a variety of phenylpropanoid compounds. Therefore, there must be a mechanism which regulates the flux of these intermediates in developing fruits so that at a certain stage of development the flow of intermediates is directed in a particular direction leading to the synthesis of capsaicin.

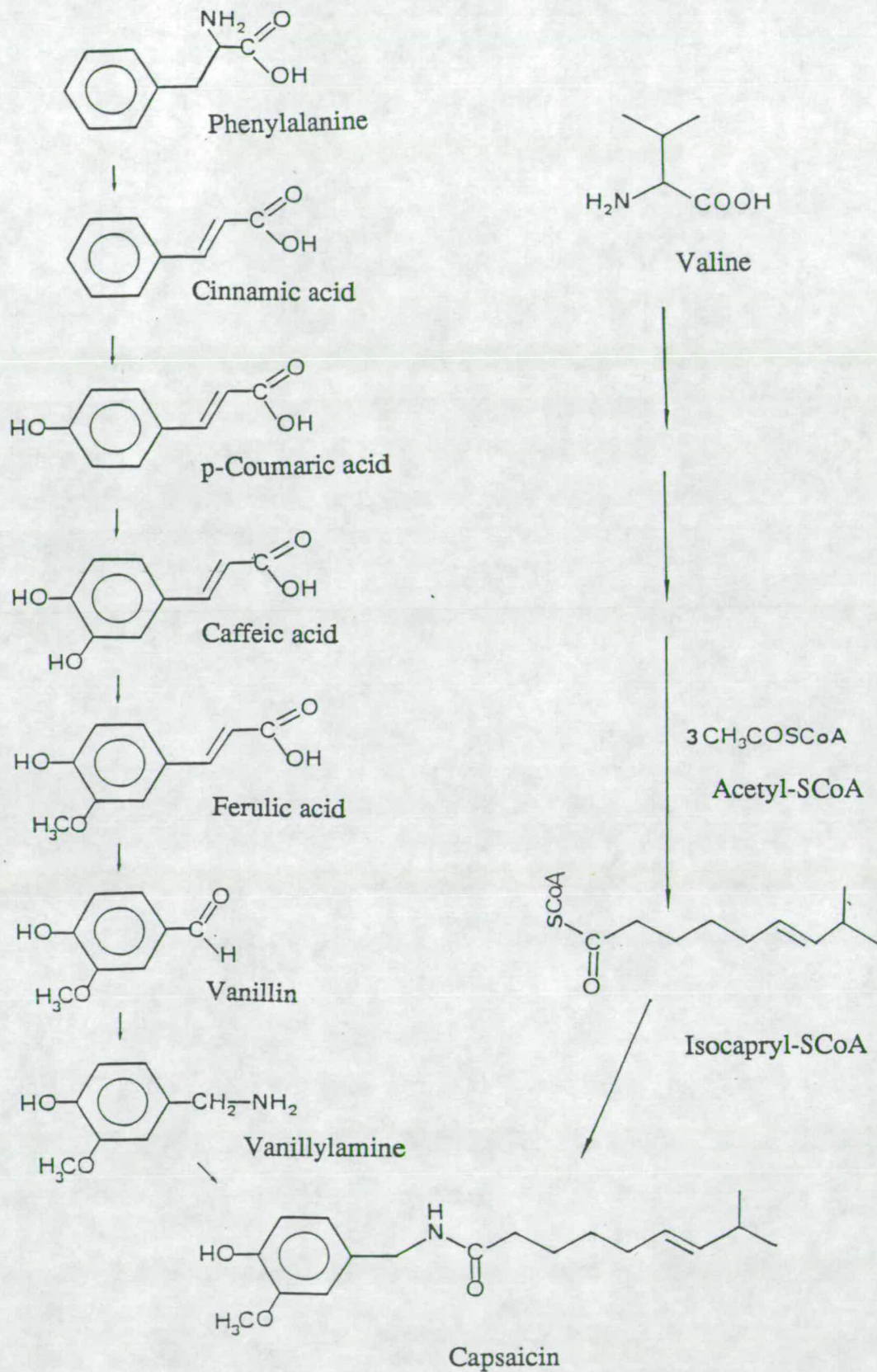


Fig. 1.1. Proposed biosynthetic pathway of capsaicin

Phenylpropanoid metabolism in higher plants

Phenylpropanoids in higher plants are derived from phenylalanine or tyrosine (Hanson and Havir, 1979). These two aromatic amino acids are produced from phosphoenolpyruvate and erythrose-4-phosphate which are intermediates in the metabolism of glucose in primary metabolism, via the shikimate pathway (Floss, 1979). This pathway operates in microorganisms and plants, but not in animals. Apart from being substrates for phenylpropanoid metabolism, phenylalanine and tyrosine are also precursors for protein in primary metabolism and it has been suggested that two pools exist for phenylalanine in which one pool provides a precursor for the synthesis of protein and the other for the synthesis of phenylpropanoid compounds (Oak & Bidwell, 1970 and Hall *et al.* 1986).

Two enzymes responsible for bridging primary metabolism (protein synthesis) and secondary metabolism (phenylpropanoid synthesis) are phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), two enzymes with a key role in general phenylpropanoid metabolism. PAL and TAL activity were first demonstrated in barley by Koukol and Conn in 1961 and since then there have been numerous reports of the occurrence of these enzymes in a variety of species. The ratio of PAL to TAL in different species varies, with PAL always present while TAL may be absent. A relatively high proportion of TAL has been reported in cereals and grasses (Young *et al.*, 1966; Camm and Towers, 1973; Jangaard, 1974). However, in fruits of *Capsicum frutescens* PAL seems to be present at a much higher level than TAL as shown by the much lower incorporation of radiolabelled tyrosine into capsaicin than phenylalanine (Bennet and Kirby, 1968). PAL activity leads to the production of metabolites, such as cinnamates and their corresponding aldehydes and alcohols, benzoates and their corresponding aldehydes and alcohols, flavonoids, coumarins, lignins, lignans, tannins and styrenes (Manitto, 1981) (see Fig. 1.2).

i. Cinnamates, cinnamaldehydes and cinnamylalcohols

Cinnamic acid, the product of PAL activity in higher plants, undergoes a subsequent hydroxylation and methylation to produce the core metabolites in

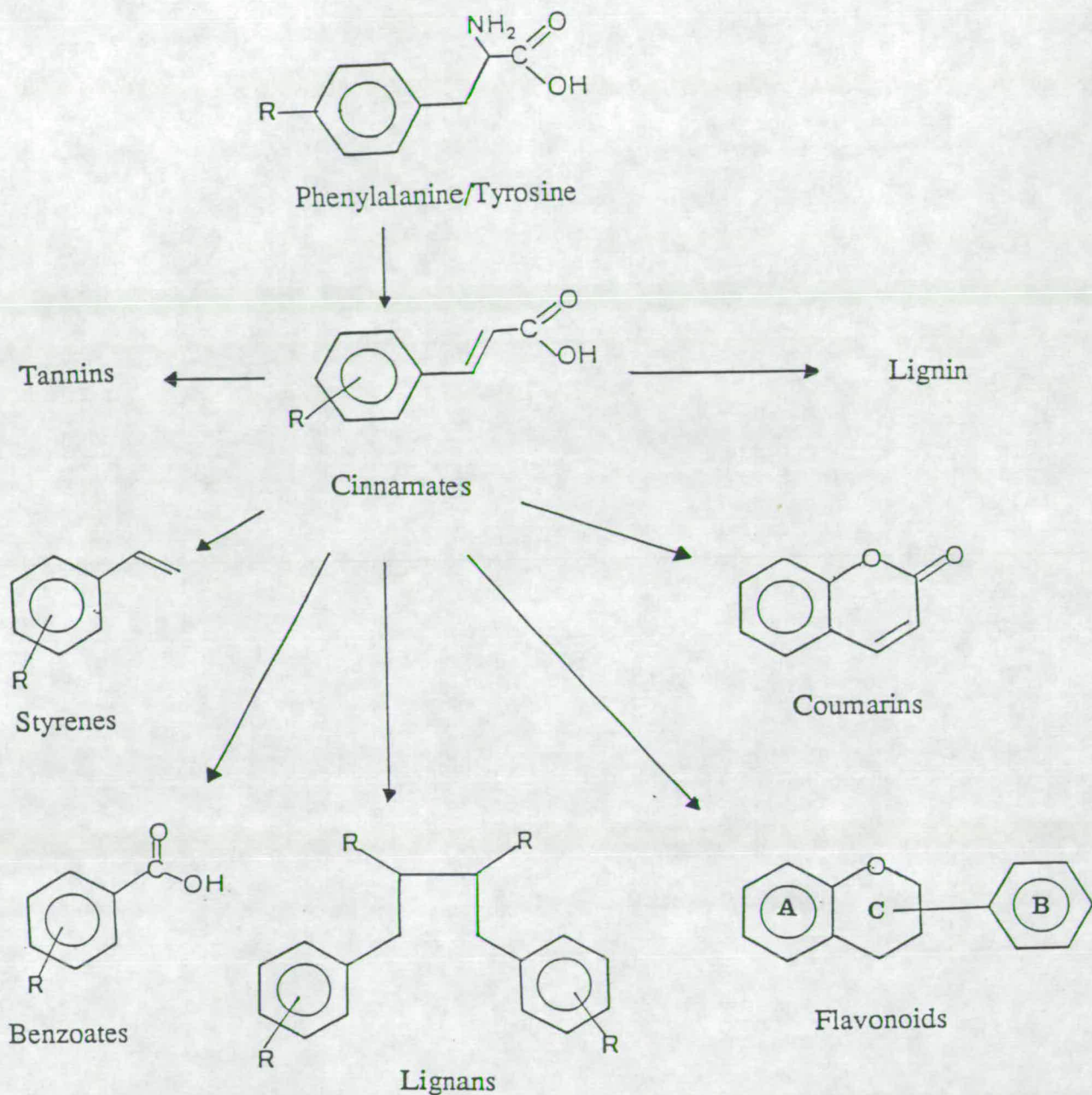


Fig. 1.2. The variety of secondary metabolites produced from phenylalanine or tyrosine in higher plants. In addition, there are several other metabolites derived from phenylalanine, but their occurrence is very limited and therefore are not included in this simplified scheme.

phenylpropanoid metabolism. These are p-coumaric, caffeic, ferulic and sinapic acid (Hanson and Havir, 1979). The enzymes involved in these reactions are cinnamate 4-hydroxylase, p-coumarate 3-hydroxylase and catechol O-methyl transferase. Cinnamate 4-hydroxylase, which requires molecular oxygen, NADPH and 2-mercaptoethanol for activity and has an optimal activity at pH 7.5, has been characterised by Russel (1971) and Russel & Conn (1967) in pea seedlings. Characterization of p-coumarate 3-hydroxylase, which catalyses the conversion of p-coumaric to caffeic acid has been carried out by Vaughan and Butt (1969); this enzyme requires molecular oxygen and a reductant such as ascorbate, tetrahydrofolate, NADH or NADPH (Vaughan and Butt, 1969; Barlett *et al.*, 1972). The activity of this enzyme is increased by excision and illumination (Bowell and Butt, 1983). Methylation of caffeic acid at the 3 position to produce ferulic acid is catalysed by catechol methyl transferase (Poulton, 1981) with S-adenosylmethionine (SAM) as methyl donor which undergoes conversion to S-adenosyl homocysteine (SAH) after releasing its methyl group. This enzyme has been characterized in a number of gymnosperms and angiosperms (Poulton, 1981). Sinapic acid can be produced from 5-hydroxyferulic acid (Grand, 1984) or 3,4,5-trihydroxycinnamic acid (Poulton *et al.*, 1976a,b), catalysed by the same enzyme as that for the methylation of caffeic acid. Fig. 1.3. shows the pathway leading to the formation of core metabolites in phenylpropanoid metabolism in higher plants and the enzymes catalysing these core reactions.

These core metabolites are metabolized actively in plants and are rarely found as free acids. These compounds are usually accumulated as esters with sugars, quinic acid, shikimic acid or glycerol (Pridham, 1965; Harborne, 1979; Gross, 1981; Shimomura *et al.*, 1987). Complex structures of conjugated cinnmates in which more than one molecules of cinnamate and sugar form a single compound have also been reported frequently (Callis *et al.*, 1988, Shimomura *et al.*, 1988). p-Coumaric and ferulic acid also form ester links with cell wall polymers (Hartley, 1972,1986; Fry, 1982, 1984, 1987).

Cinnamaldehydes and cinnamylalcohols are reduction products of cinnamic acids

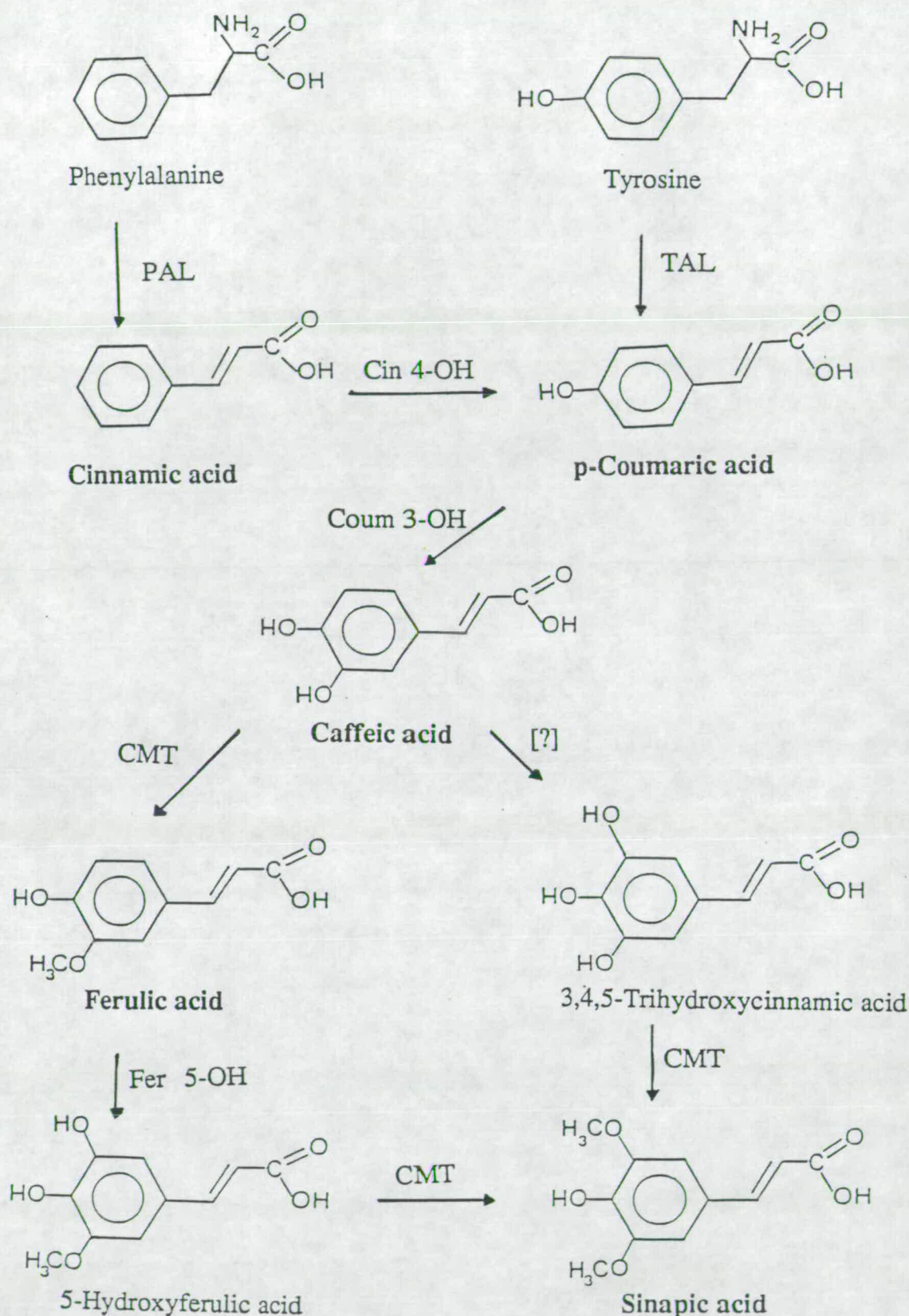


Fig. 1.3. The pathway leading to the formation of core metabolites [bold] in phenylpropanoid metabolism in higher plants and the catalysing enzymes. PAL: Phenylalanine ammonia lyase, TAL: tyrosine ammonia lyase, Cin 4OH: cinnamate 4-hydroxylase, Coum 3-OH: p-coumarate 3-hydroxylase, Fer 5-OH: ferulate 5-hydroxylase, CMT: catechol methyltransferase, [?]: has not been characterized.

and these groups of compound are intermediates in lignin synthesis. It has been established that the activation of the acyl groups of cinnamic acids by the formation of cinnamoyl-S-CoA esters is required for the reduction to take place (Ebel and Grisebach, 1973; Rhodes and Woollorton, 1974). Cinnamaldehydes are rarely accumulated, but cinnamylalcohol glycosides such as coniferin (glucoside of coniferylalcohol) and syringin (glucoside of sinapylalcohol) and 4-cinnamylalcohol- β -D-glucose are commonly found in gymnosperms but not in angiosperms (Brown, 1966; Hosel and Todenhagen, 1980).

ii. Benzoic acids, benzaldehydes and benzylalcohols

Benzoic acids are produced in microorganisms from shikimic, dehydroshikimic or chorismic acid, intermediates in the shikimate pathway (Pridham, 1965; Manitto, 1981; Haslam, 1986). In higher plants these compounds are synthesized either from cinnamic acids by β -oxidation or by aromatization of shikimic or dehydroshikimic acid. Benzoic acid is only produced from cinnamic acid (Zaprometov, 1978) while gallic acid (3,4,5-trihydroxybenzoic acid) is synthesized mainly from shikimic and dehydroshikimic acid (Zaprometov, 1978; Haslam, 1986, 1989). Benzaldehydes and benzylalcohols are reduction products of benzoic acids. The same mechanism proposed for the formation of cinnamaldehydes and cinnamylalcohols from cinnamic acids is possibly involved in these reactions.

Benzoic acid, benzaldehyde and benzylalcohol can be present in the free form in plants. Benzoic acid is one of the components of resin obtained from *Styrax benzoin* (*Styracaceae*). Benzaldehyde and benzylalcohol are present as components of volatile oil from bitter almond and jasmine respectively (Badavari *et al.*, 1989). Their hydroxy derivatives, on the otherhand, are mainly accumulated as conjugates. Hydroxybenzoic acid can occur as esters or glycosides while hydroxy benzaldehydes and hydroxy benzylalcohols are mainly present as O-glycosides (Harborne, 1984; Haslam, 1986). Indeed, vanillin (3-methoxy-4-hydroxybenzaldehyde) from the bean of *Vanilla planifolia* is present as a glucoside in fresh mature fruits and is then released from this conjugate during the curing process (Narbard *et al.*, 1990).

iii. Flavonoids

Flavonoids are a group of compounds with a 2 or 3-phenylchromone as the base structure (see Fig. 1.2). Their presence in plants can be easily recognized from their colour, usually as pigments of flowers, fruits and leaves. Various flavonoids are sub-grouped according to the oxidation state and substitution pattern of ring C and the position of ring B attached to ring C (Hahlbrock, 1981). According to their distribution, flavonoids may be divided into major and minor flavonoids. Flavones, flavonols, anthocyanins and anthocyanidins are widely distributed in higher plants and constitute the major flavonoids, while chalcones, aurones, flavanones, dihydrochalcones and isoflavones, due to their limited distribution are placed in the minor flavonoids. Flavonoids are usually present as glycosides. The glycones are often further substituted with acyl, malonate, p-coumarate, caffeate or ferulate (Hahlbrock, 1981). Conjugation of flavonoids with sulphate has recently been reviewed by Barron *et al.* (1988). Flavonoids are derived from cinnamic acid and three molecules of acetate. The biosynthesis of flavonoids has been studied intensively in soya bean and parsley cell cultures and has been reviewed by Hahlbrock (1977, 1981); Hahlbrock and Grisebach (1979); Hahlbrock and Scheel (1989); Grisebach (1985). Sixteen enzymes involved in the biosynthesis of flavonoid glycosides in parsley cell cultures have been characterized (Hahlbrock, 1981) and the mRNAs coding the PAL, 4-coumarate SCoA-ligase and chalcone synthase have also been identified (Hahlbrock and Scheel, 1989). It was observed that the active synthesis of these mRNAs precedes the increase in the activities of the corresponding enzymes, indicating the control of the flavonoid synthesis is at the transcription step.

iv. Lignin

Lignin is a polymerization product of hydroxycinnamylalcohols involving the formation of C-C and C-O bonds. In plant cells, lignin forms covalent bonds with polysaccharides of the cell wall (Gross, 1979; Grisebach, 1981; Obst and Kirk, 1988; Azuma and Tetsuo, 1988). Lignin is one of the components of the secondary cell wall which is deposited ^{and into} onto the primary cell wall after the cell stops growing (Hall *et al.*, 1982) and is responsible for the physical rigidity of the cells. Lignin is the most

abundant secondary metabolite on earth and contributes 20 to 35% of the dry weight of woody tissue (Zaprometov, 1978; Gross, 1979). It is also present in cell cultures, especially those which are grown on a medium in which the cells tend to form large aggregates (Hahlbrock, 1977).

Lignin is synthesized from phenylalanine via p-coumaric, ferulic and sinapic acid. These three acids are activated by hydroxycinnamoyl-SCoA ligase, which then undergo a subsequent reduction to produce the corresponding hydroxycinnamylalcohols (lignin monomers) (Hahlbrock, 1977; Hahlbrock and Grisebach, 1979; Gross, 1979; Grisebach, 1981). The last step in the biosynthesis of lignin is a polymerization which is catalysed by peroxidase (Gross, 1979; Grisebach, 1981).

v. Tannins

Tannins are groups of compounds responsible for the astringent taste in unripe fruits, they are polymers of phenolics. Their occurrence in plants, however, is not limited to fruits, as they are also present in other parts of the plant. As with lignin, tannins are also polymers of phenolic compounds, but these polymers are readily soluble in polar solvents such as water, methanol or ethanol. Tannins are divided into condensed tannins (proanthocyanidins) which upon hot acid treatments produce a red precipitate, and hydrolysable tannins (gallotannins and ellagitannins), which are hydrolysed in hot acid releasing glucose and gallic or ellagic acid. Proanthocyanidins have a flavan skeleton, and are therefore derived from cinnamates together with three molecules of acetate, while the gallotannins and ellagitannins, which are polymers of glucose esters of gallic and ellagic acid respectively, are synthesized from shikimic and dehydroshikimic acid (Haslam, 1986, 1989).

vi. Other compounds derived from phenylpropanoids

Apart from the groups of compounds already described, there are still several groups of secondary metabolites derived from phenylalanine or tyrosine with a restricted distribution in higher plants. These are coumarins, lignans and styrenes.

Coumarins (benzopyrones) are a product of the lactonization of o-coumaric acid and are formed from cinnamic acid following o-hydroxylation, O-glucosylation, *trans-cis* isomerization and lactonization (Brown, 1979, 1981). For 7-oxycoumarins, p-coumaric acid is a more favourable precursor than o-coumaric acid (Austin and Meyers, 1965). Coumarins are mostly found in *Rutaceae*, *Umbelliferae*, *Leguminosae* and *Compositae* (Dreyer, 1986). Lignans are dimers of two phenylpropane units linked by at least one C-C bond between carbon atom at the 2-side chains of both monomers. These compounds are widely distributed in plants (Pelter, 1986), but most of the lignans already reported have been found in *Magnoliaceae* and *Piperaceae* (Manitto, 1981). Styrenes are groups of C₆-C₂ compounds which are probably synthesized by decarboxylation of cinnamic acids. These compounds are mainly found in microorganisms (Manitto, 1981).

Phenylpropanoid metabolism in fruits and cell cultures of *Capsicum frutescens*

The accumulation and synthesis of capsaicin in *Capsicum* has been investigated by a number of workers, but the relationship between phenylpropanoid metabolism and capsaicin synthesis has only been studied in this laboratory (see Hall *et al.*, 1986, 1987; Holden *et al.*, 1987; Yeoman *et al.* 1989; Holland, 1989 and Hall & Yeoman, 1991). In this study most of the work has been carried out with fruits and to a lesser extent with cell cultures in order to acquire a better understanding of the regulation of capsaicin synthesis and the part played by a variety of compounds derived from cinnamate.

Cinnamate, p-coumarate, caffeate and ferulate are the core metabolites in phenylpropanoid metabolism but are present at vanishingly low concentrations in both cell cultures and fruits. These compounds have only been detected by TLC-autoradiography after labelling with [U-¹⁴C]-phenylalanine or [3-side chain-¹⁴C]-cinnamic acid (Hall *et al.*, 1986, 1987; Holden *et al.*, 1987a, Hall and Yeoman, 1991). However, several unknown free phenolics are present in cell cultures of particular cell lines and these are also released to the bathing medium (Lindsey, 1986b; Holden, 1989). Most phenolics in cell cultures and fruits are bound

to cell wall polymers (Hall *et al.*, 1986, 1987; Holden *et al.*, 1987a,b) and this group of phenolics constitutes a major sink in phenylpropanoid metabolism in cell cultures (Hall and Yeoman, 1991).

The initial nitrate concentration in cell cultures greatly affects the synthesis of cell wall bound phenolics. A decrease in the synthesis of cell wall bound phenolics was observed in cell cultures grown under conditions of nitrate limitation (Hall *et al.*, 1986, 1987; Holden *et al.*, 1987a). Cell immobilization also affects the balance sheet of phenolic synthesis in cultures. Apart from increasing the synthesis of capsaicin, cell immobilization also increases the synthesis of soluble phenolics (Lindsey, 1986b) despite the lower levels of PAL and cinnamate 4-hydroxylase activity in this system when compared with freely suspended cells (Holden *et al.*, 1987a). The higher cell-cell contact in immobilized cells (Yeoman, 1987) could possibly make the enzyme work more efficiently. Another possibility is that the synthesis of the major products of phenylpropanoid metabolism is reduced in immobilized cells. Indeed, the synthesis of lignin was observed to decrease after the addition of sinapic acid to immobilized cell cultures (Hall and Yeoman, 1991).

Phenolic intermediates of capsaicin biosynthesis have been added to cell cultures and fruits. These compounds are rapidly converted to the immediate precursor and the product (Yeoman *et al.*, 1989). Conversion of cinnamate to phenylalanine was observed by Hall *et al.* (1986), but phenylalanine produced from cinnamate is not incorporated into protein in fruits (Hall *et al.*, 1986, Holden *et al.*, 1987a). This led to the suggestion of two phenylalanine pools, one of which is specifically for the provision of precursors for phenylpropanoid metabolism. It was also observed that vanillylamine added to fruits and cell cultures is partly converted to vanillin (Holland, 1989).

The metabolic role of the variety of cinnamate derived compounds shown to be present in cell cultures and fruits has been investigated in this study especially in relation to the synthesis of capsaicin.

Aims and Objectives

The aim of this study was to determine the relationship between the metabolism of a variety of secondary metabolites derived from cinnamic acid (phenylpropanoid and related compounds) and the synthesis of capsaicin in *Capsicum frutescens*. This aim can be achieved by fulfilling the following objectives.

1. Identification of the phenolic compounds (free, esters, glycosides, bound) accumulated in callus, suspension cultures and fruits.
2. Establishment of the pattern of accumulation of these phenolic compounds in cell cultures over a culture cycle and in developing fruits.
3. Definition of the pattern of incorporation of radioactively labelled cinnamic, p-coumaric and caffeic acid into the phenolics in fruits before, during and after the onset of capsaicin synthesis.
4. Establishment of the synthetic route and metabolic fate of hydroxycinnamoyl glycosides in fruits.

Originally, it was planned to perform all of these investigations with cell cultures, but capsaicin was poorly accumulated in the cell cultures used in this study. Therefore, most of the biosynthetic studies were carried out with fruits.

Chapter 2: MATERIALS AND METHODS

2.1. Plant Materials

The species used throughout this study was the chilli pepper *Capsicum frutescens*. Plants of *Capsicum frutescens* Mill. var. Cayenne, used for preliminary experiments, were grown under glass at a minimum temperature of 16°C, in a 16h. day maintained with a supplementary 400 Watt mercury lamp, light intensity was variable due to environmental factors. Plants used for detailed experimental studies were grown in a constant environment chamber under controlled conditions, temperature 25±1°C, 16h. day, light intensity 135µmol.m⁻²sec⁻¹ (Thorn warmwhite fluorescent, Phillips Tungsten).

Seeds were sown in 5cm pots and after three weeks seedlings were repotted into 15cm pots, using John Innes compost No 2. Flowers appeared six weeks after sowing. In order to record fruit age, individual flowers were labelled on the date of anthesis (see Fig. 2.1). Watering was carried out as necessary to keep the soil moist.

2.2. Tissue and Cell Cultures

2.2.1. Preparation of media

Cultures were maintained on Murashige and Skoog (MS) (1962) or Schenk and Hildebrandt (SH) medium (1972). These media were supplied by Imperial Laboratories, U.K. To these basal media, 3% sucrose (w/v), 0.1mg.l⁻¹ kinetin, 1.0mg.l⁻¹ p-chlorophenoxyacetic acid (CPA) and 0.5mg.l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) were added. The pH of the medium was adjusted to 5.8-6.0 with N HCl or N KOH. Bacterial agar No 1 (Oxoid) (10g.l⁻¹) was added to prepare solidified media.

2.2.2. Sterilization and aseptic work

Glassware and culture media were sterilized by autoclaving at 121°C for 20min. Plant tissue was surface sterilized by immersing explants in 2% (w/v) sodium



Fig. 2.1. Plant of chilli pepper *Capsicum frutescens* Mill. CV. Cayenne grown in a constant environment chamber with labelled fruits

hypochlorite containing three drops of Tween 80 for 10min. followed by rinsing with sterile distilled water three times. Before sterilization, the cut ends of explants (internodes) were sealed with wax (Paraplast-Lances, Eire) to protect the tissue from sodium hypochlorite. Aseptic manipulations were carried out in a laminar air flow cabinet. The cabinet walls were sprayed and swabbed with absolute ethanol before and after aseptic work. All instruments used for manipulating cultures were stored in ethanol and flamed before use.

2.2.3. Initiation and Maintenance of Cultures

i. Callus cultures

Callus cultures were initiated from internodes of plants. Cut internodes (2-3cm in length) were sterilized as described in 2.2.2. After sterilization these internodal pieces were cut into 0.5cm lengths and placed onto 20-25ml agar medium in 9cm Petri dishes (Sterilin). The dishes were sealed with parafilm (American Can Company) to exclude contaminants and prevent desiccation of the medium. Cultures were kept in a room under strictly controlled conditions, temperature $25\pm 1^{\circ}\text{C}$, light intensity $25\mu\text{mol.m}^{-2}\text{sec}^{-1}$ photon flux density (Compton warmwhite fluorescent tubes). Callus grown on MS and SH media ^{was} were maintained by transferring 3-4 pieces of c 250mg weight callus onto fresh medium every three weeks.

ii. Suspension cultures

After several subcultures, calluses became friable, especially those grown on SH medium. To initiate suspension cultures, friable callus was separated out gently with a spoon spatula and placed in a sterile Petri dish. Approximately 2.5-3.0g of this callus was transferred to 50ml of liquid medium in a 250ml Erlenmeyer flask and covered with a double layer of aluminium foil. Suspension cultures were kept under the same conditions as callus cultures but in addition were agitated on an orbital shaker at a speed of 98rpm at an amplitude of 8mm. Stock cultures were maintained by transferring approximately 2.5g cells with a spoon spatula into fresh medium.

The interval of subculturing was two and three weeks for cultures grown in SH and MS media respectively.

2.3. Analytical Methods

2.3.1. Measurement of Growth Parameters

2.3.1.1. Determination of fresh weight

The fresh weight (FW) of suspended cells was determined after they had been filtered through damp Whatman No.1 filter paper (Whatman International Ltd., Maidstone, U.K.). Callus tissue was removed from agar and cleaned from any residual medium. All cells were weighed on a Sartorius type 1412 balance.

2.3.1.2. Determination of dry weight

Dry weight (DW) was determined by drying a known fresh weight of cells or tissue in an oven at 90°C for 24h. Samples were cooled in a desiccator and weighed on an analytical balance (Oertling).

2.3.1.3. Determination of cell number

Cell number was determined according to the method described by Reinert and Yeoman (1982). Approximately 500mg of cell aggregates from suspension cultures or from callus were macerated in 2ml (10% w/v) chromic acid (solution of chromium trioxide in distilled water) for 15h. at room temperature. Aggregates of cells were further gently separated and dispersed with the aid of a Pasteur pipette. Cell number was determined using a Hawksley crystalite Haemocytometer (1.8 µl). A total of six grids were counted and the mean used to calculate the number of cells in culture. Dilution of the cell suspension was carried out to give a cell density of 150-200 cells *per* grid.

2.3.1.4. Determination of length and fresh weight of fruits

The length and fresh weight of fruits was measured post harvest, excluding calyx and stalk.

2.3.2. Extraction and Fractionation of Phenolic Compounds

2.3.2.1. From the tissue

Tissue (1-10g FW) was ground in a mortar containing sand, with methanol (MeOH) (at least 10ml.g⁻¹). The mixture was transferred to a glass bottle with a screw top containing a magnetic stirring bar, the solution was saturated with nitrogen and stirred at room temperature, in the dark for 24h. The extract was filtered using a Millipore funnel fitted with a glass microfibre filter (Whatman GFC) and the residue washed 2X with 25ml MeOH. Combined filtrates were evaporated under reduced pressure at 40°C, the extract residue was resuspended in 50ml water and acidified with three drops of glacial acetic acid (HOAc). Free phenolics were extracted from this aliquot 3X with equal volumes of 50ml ethyl acetate(EtOAc)-diethylether(Et₂O) (1:1). The combined fractions of organic phases were dried with anhydrous sodium sulphate and evaporated under reduced pressure at 30°C. The dried extract was redissolved in 1.0ml of HPLC grade MeOH, unless otherwise stated, and stored at -20°C for further analysis. The water fraction containing the glycosylated phenolics was evaporated to dryness under reduced pressure at 45°C, then unless otherwise stated, redissolved in 1.0ml of 80% HPLC grade MeOH in water for further analysis. The cell residue was further treated with 0.5N NaOH at room temperature for 24h. to saponify cell wall phenolics. The mixture was filtered through a glass microfibre filter. If necessary, the saponifiable cell wall phenolics were further fractionated into EtOAc-Et₂O (1:1) from the filtrate after acidification with 6N HCl to pH 3. Lignin which was not extracted in the above treatment, was removed from the dried cell residue with 25% acetylbromide (further described in 2.3.5.2). Fig. 2.3.1 summarizes these extraction and fractionation procedures.

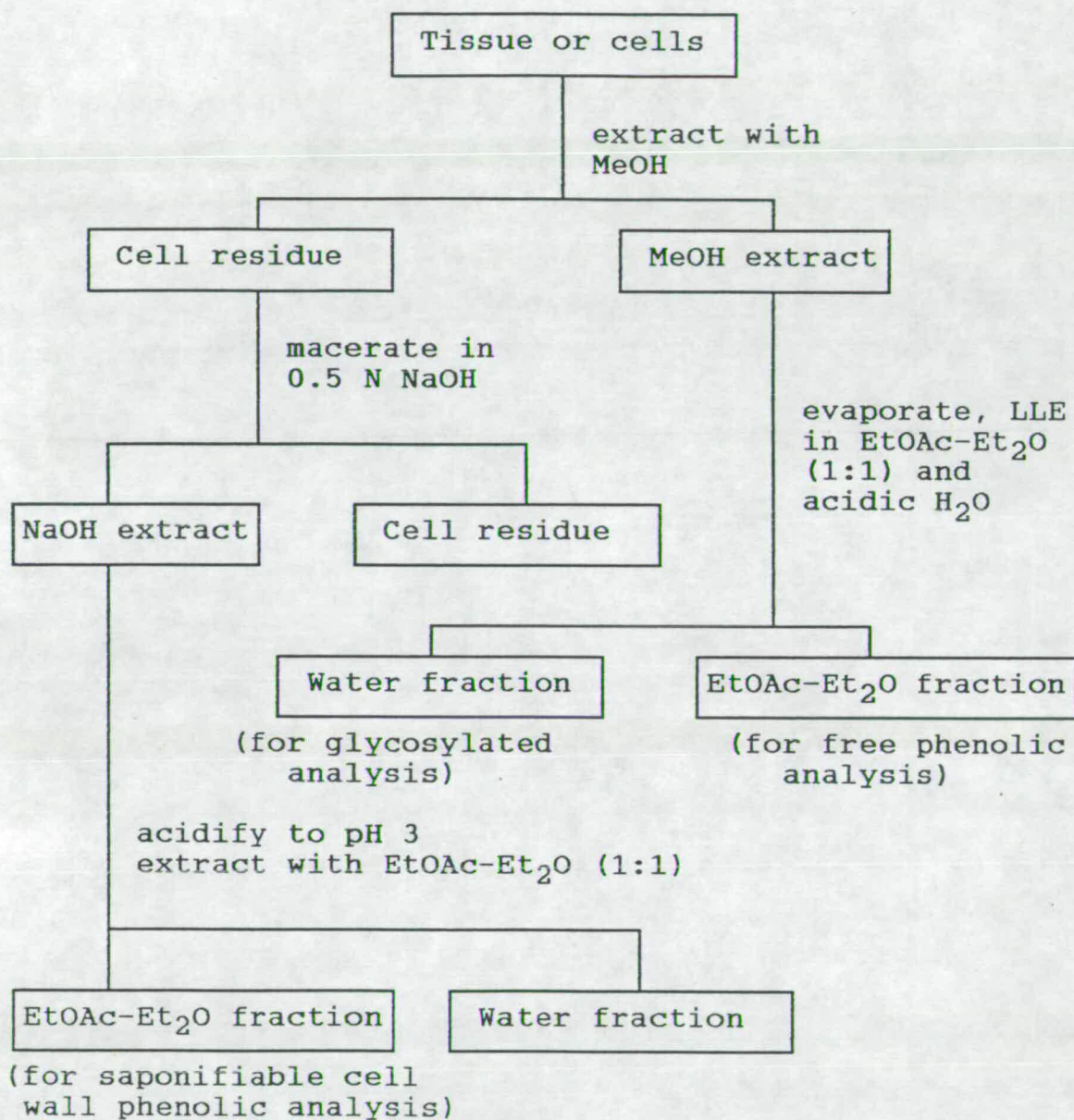


Fig.2.3.1.Extraction and fractionation scheme of phenolic from plant and callus tissue. LLE: liquid-liquid extraction.

2.3.2.2. From suspension culture medium

Most of the phenolics released into the medium in suspension cultures were in a free form. These phenolics were extracted from the medium after filtration of cultures through a Buchner funnel fitted with Whatman No.1 filter paper (Whatman). Phenolics were extracted by the procedure given for free phenolics in 2.3.2.1.

2.3.3. Thin Layer Chromatography

2.3.3.1. Adsorbents and solvent systems

For most analytical purposes, plastic backed TLC plates coated with silica gel 60 (E. Merck, West Germany) were used, while for preparative scale separations, glass backed TLC plates coated with K5 Silica gel (Whatman) were employed. The solvent systems used to separate free phenolics were solvent system-I: benzene(C_6H_6)-HOAc (90:20) or solvent system-II: $CHCl_3$ -EtOAc-HOAc (50:50:1). Glycosylated phenolics were separated using solvent system-III: EtOAc-EtOH-HOAc- H_2O (15:5:1:3). Sugars (glycones) were separated on aluminium backed TLC plates coated with cellulose MN (E. Merck, West Germany) using double developments in the same direction. The first elution was in solvent system-IV: butanol($BuOH$)-HOAc- H_2O (3:1:1) and the second was in solvent system-V: EtOAc-pyridine- H_2O (10:4:3) (Fry, 1988). Other solvent systems were also used to separate simple mixtures containing two or three components.

2.3.3.2. Visualization of separated compounds

i. Long wavelength ultraviolet light

Some phenolics especially hydroxycinnamates, such as ferulic acid, caffeic acid, sinapic acid and their conjugates fluoresce under long wavelength (365nm) ultraviolet (UV) light. These compounds can be easily visualized on TLC under UV light in a dark room or cabinet.

ii. Iodine fumes

Fumes of iodine are a general visualizing agent for those compounds with a double bond. The reaction of iodine with most phenolics is not reversible as a substitution reaction can take place. However, its reaction with cinnamic acid is reversible, since it has no hydroxy phenolic group. This reagent is suitable to detect cinnamic acid which does not react with other general phenolic reagents. There is no loss in counting efficiency when levels of radioactivity are measured. The compounds on TLC were visualized by exposing the plates in a sealed TLC tank containing saturated iodine fumes.

iii. Potassium permanganate

Potassium permanganate is another general visualizing reagent. All phenolics could be detected using this reagent. It was used especially to detect cinnamic acid if staining with iodine was found to be unsatisfactory. The reagent was made by dissolving 153mg potassium permanganate and 15.3mg sodium carbonate in 10ml water.

iv. Diazo reagent (Miedziak & Waksmundski, 1983)

The diazo reagent stains phenolics substituted at different positions giving a range of colours when cellulose is used as the adsorbent. The reagent was made up by mixing 5.0ml (1% w/v) sulphanilamide in 10% v/v HCl, 5.0ml (5% w/v) sodium nitrite and 50ml MeOH. Some phenolics can be detected by spraying with this reagent only, but most appear after spraying with the second reagent (10% w/v sodium carbonate).

v. Ferric chloride - potassium ferricyanide

This reagent was freshly prepared prior to staining by mixing equal volumes of 1% (w/v) ferric chloride and 0.5% (w/v) potassium ferricyanide. After ^{development} elution the plate was dried then sprayed with the reagent. Phenolics give a blue to reddish colour

with this reagent. The higher the level of hydroxylation, the more intense the colour produced.

vi. Aniline hydrogen phthalate reagent

This reagent was used to detect sugars on aluminium backed TLC plates. The reagent was prepared freshly each time by mixing 100ml of a stock solution containing 16g phthalic acid, 490ml acetone, 490ml ether, and 20ml water with 0.5ml aniline. The plate was dried then sprayed with the reagent and heated for 5min. at 105°C.

2.3.4. High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) was used for the separation, identification and quantification of phenolics in extracts. It was also used for small scale isolation of separated phenolics.

2.3.4.1. Chromatographic conditions

The chromatograph was a Hewlett-Packard HP1090 Liquid Chromatograph, with an HP1040 diode array detector, fitted with an autosampler and binary solvent delivery system. Separation was achieved using a reverse phase 5µm Hypersil MOS(C-8) (20X0.5cm) column (Phase Separation, Clwyd, U.K.), UV detection at 280nm, solvent system with a gradient of 5% HOAc and MeOH as described by Hall *et al.* (1987) and a flow rate 1.0ml.min⁻¹. Injection volumes ranged from 5 to 20µl and an oven temperature of 40°C was used. Slight modifications to the gradient of the solvents, detection wavelength and the time of elution were employed as necessary. Compounds eluted from HPLC were collected using a LKB-221 Superrac Fraction Collector (LKB, ^PUppsala, Sweden). This was carried out if further analysis such as identification or radioactive measurement was required.

2.3.4.2. Sample preparation

Samples for HPLC analysis were dissolved in 1.0ml of 100% or 80% HPLC

grade MeOH, unless otherwise stated. Prior to injection, samples were filtered using a microfilter (Bioanalytical System), fitted with a 0.4µm nylon-66 filter (Rainin) and by centrifugation at 1000Xg for 5min. Filtrates were then transferred into 2.0ml autosampler vials (Phase Separation, Clwyd, U.K.) and sealed with a crimp top.

2.3.4.3. Preparation of HPLC solvents

The mobile phase was composed of two stock solvents, MeOH and 5% HOAc. To remove any particulate matter present in the solvents, MeOH was filtered through a 0.45µm pore Nylon-66 membrane (Anachem, Luton, U.K.) and the aqueous solvent was filtered through a 0.45µm pore cellulose acetate membrane (Schleicher and Schull, Surrey, U.K.). Filtered solvents were then degassed to remove suspended air by gently bubbling helium through them for 10min.

2.3.5. Estimation of Phenolic Levels

2.3.5.1. Phenolics separated on HPLC

Authentic markers of free phenolics were used as standards in the determination of the levels of phenolics in extracts. Two water soluble conjugated phenolics were isolated from a mixture of mature fruits of *Capsicum frutescens* in mg quantities. These were 3-rhamnosylquercetin and 7-glucosylluteolin and were also used as standards. The calibration curves between peak areas and the amounts of these phenolics are presented in Fig. 2.3.2.

The aglycones of some glycosylated phenolics were identified and the molar levels of these compounds in extracts were estimated from the calibration curves of the corresponding aglycones. Comparison between the relative amounts of unknown phenolics in different samples was based on the peak areas of compounds.

2.3.5.2. Lignin

Lignin content was determined spectrophotometrically following solubilization of lignin with 25% (v/v) acetylbromide in HOAc as outlined below (Johnson *et al.*,

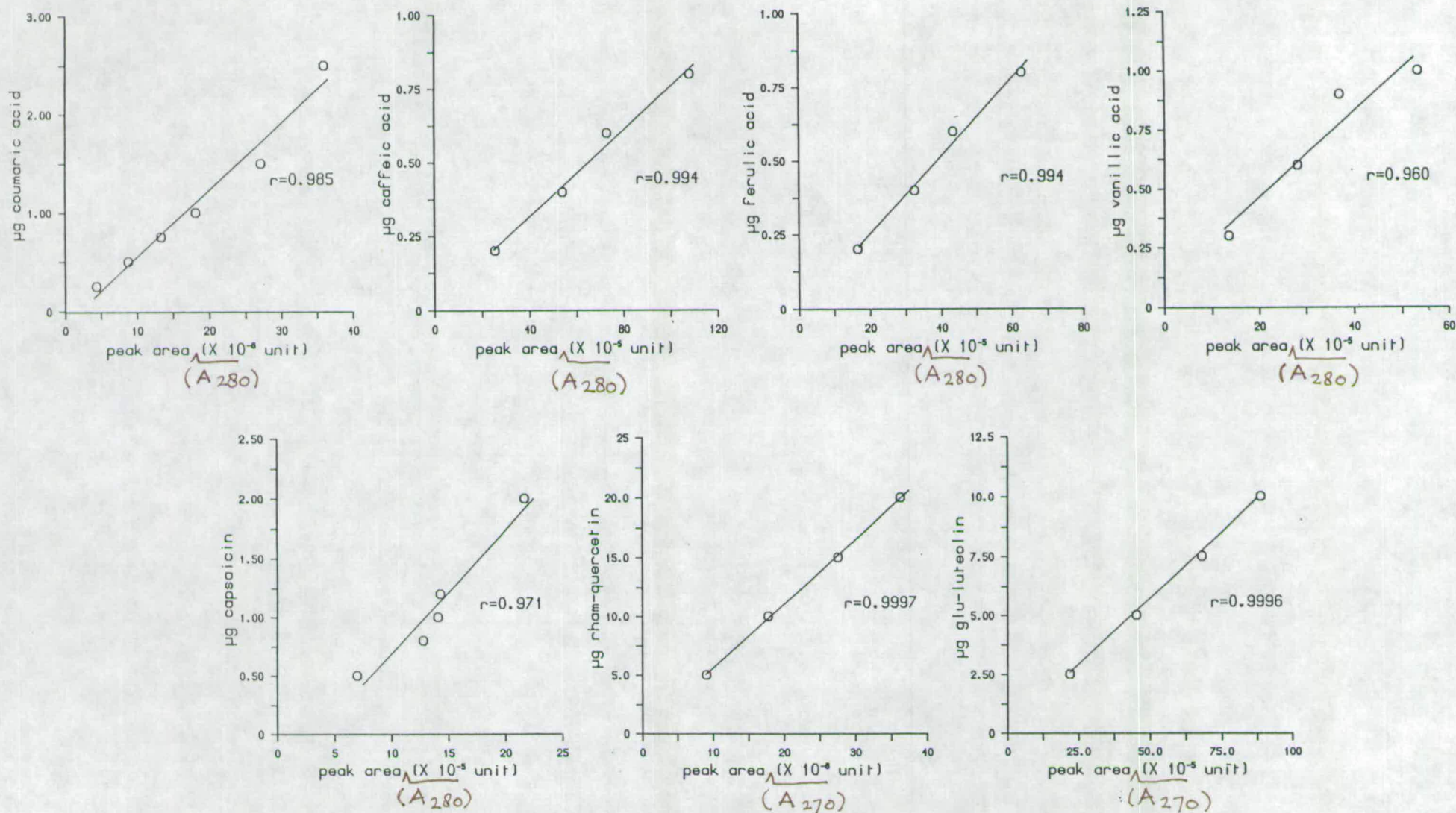


Fig. 2.3.2. Correlation between peak areas (obtained from HPLC chromatograms) and amount (μg) of several phenolics, rham-quercetin: 3-rhamnosylquercetin and glu-luteolin: 7-glucosylluteolin.

1961, Morrison, 1972).

The cell residue (30mg) was placed in a 15ml screw top tube and 1.5ml of 25% acetylbromide was added. The mixture was then heated in a water bath at 70°C for 30min. with the top unscrewed and gently shaken every 10min. The tube was then cooled to room temperature. and 1.3ml of 2N NaOH was added to neutralize excess acetylbromide. To this mixture 2ml HOAc was added and the solution was transferred into a 25ml volumetric flask. Bromine and polybromide which are produced during the reaction absorb strongly at the measurement wavelength. These two compounds were removed by the addition of 0.18ml of 7.5N hydroxylammonium hydrochloride. Finally the solution was diluted to 25ml with glacial acetic acid. The time elapsed from the addition of 2N NaOH to the final dilution should not exceed 15min. The absorbance of the solution was read at 280nm with a blank treated in the same way as the sample. The lignin content was calculated from the following equation:

$$\text{lignin(g.l}^{-1}\text{)} = (A_s - A_b)/a$$

where A_s and A_b are the absorbance of sample and blank respectively and a is the absorptivity of lignin. The average absorptivity of lignin is 24 (Fry, 1988). Further dilution was made if necessary in order to obtain an appropriate A value.

2.3.6. Radiolabelling and Measurement of Radioactivity

2.3.6.1. Radiolabelling

The radioactive precursors used in this study were dissolved in 0.05M phosphate buffer pH 7.0, unless otherwise stated. Radioactively labelled precursors were delivered to fruits as 20-50 μ l solutions via a 100 μ l Hamilton syringe injected into the loculus next to the peduncle.

2.3.6.2. Measurement of radioactivity

i. TLC-Autoradiography

Autoradiography was used to locate radioactive spots on TLC plates. Autoradiographs were prepared by contacting a radioactive chromatogram to Hyperfilm-MP (Amersham, U.K.) in a 12X15 inches autoradiographic cassette (Siemens, W. Germany) in a dark room under an X-ray safelight (Ilford 914, Ilford Ltd.). The cassette was then closed and the exposure left for two weeks at -70°C. Films were allowed to reach room temperature before developing using Gevomatic 60 automatic developer (Agfa Gavaert, W. Germany).

ii. TLC-RITA

RITA (Radioactivity Intelligent Thin Layer Analyzer) was used for rapid measurements of radioactivity in one dimensional TLC. RITA scans were carried out on an Isomess IM-3000 Radio-TLC-Analyzer (Nuclear-Interface).

iii. Liquid scintillation counting

The radioactivity of samples was determined using an Inter technique SL-3000 Liquid Scintillation Counter. The scintillation cocktails used were butyl-PBD and PPO/POPOP for [^{14}C] and [^3H] labelled compounds respectively. For non-aqueous samples the scintillation fluid was 0.61% butyl-PBD or 0.5% PPO/0.05% POPOP in toluene. For aqueous samples, TritonX-100 was added in a ratio 1 part TritonX-100 and 2 parts (non-Triton) scintillation fluid. The ratio of sample to scintillation fluid was at least 1 to 10.

Spots on TLC were scraped and eluted with 100 μl MeOH or water (depending on the solubility of the compound) and scintillation fluid added. As the HPLC solvent was a gradient mixture of 5% HOAc in water and MeOH, the eluates (1ml each) were counted by adding 4ml of Triton containing scintillation fluid.

The results obtained from the counter were in CPM (counts per minute). For

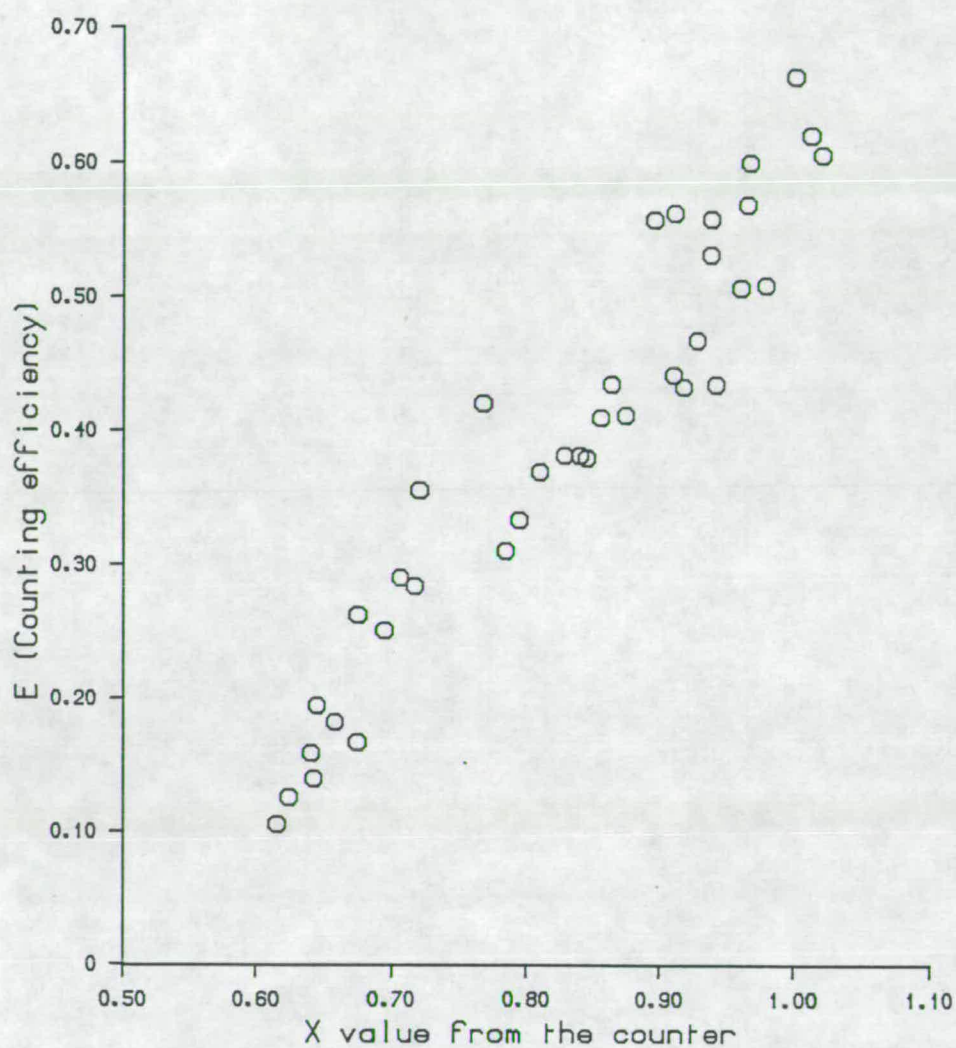


Fig. 2.3.3. Quench curve for $[^{14}\text{C}]$ labelled compounds obtained by measuring known activities of $[\text{U-}^{14}\text{C}]$ phenylalanine (Amersham) quenched at different levels with acetone.

[^{14}C] labelled compounds, these results were converted into DPM (disintegration per minute) by using the external standard ratio method. Using this method the quench curve (Fig.2.3.3) was obtained by measuring known activities of [^{14}C] phenylalanine (Amersham, U.K.) quenched at different levels by adding increasing amounts of acetone (0-300 μl into 4ml scintillation fluid). Different X (machine efficiency) values were assigned to samples and efficiencies calculated by dividing the counts obtained with the actual activities. A quench curve was plotted between X and E (efficiency) values.

2.4. Isolation and Identification of Water Soluble Phenolics from Fruits

2.4.1. Extraction and fractionation

Mature fruits (500g) were harvested from plants grown under glass (see 2.1) and homogenized in 1.0l MeOH in a blender, then left at room temperature in the dark for 24h. to enhance the extraction. The mixture was filtered through a Buchner funnel fitted with filter paper no 1 (Whatman) and washed with 500ml MeOH. Combined filtrates were evaporated under reduced pressure at 40°C. The concentrated extract was resuspended in 250ml water and extracted successively three times with equal volumes of 250ml CHCl_3 and EtOAc. The chloroform fractions which mainly contained non-polar compounds, were discarded. The EtOAc fractions, were combined and evaporated under reduced pressure at 40°C. The aqueous fraction was heated in a water bath at 70°C to remove residual EtOAc then frozen and freeze dried.

2.4.2. Isolation of Flavonoids

2.4.2.1. Flavonoid-1

The EtOAc extract was dissolved in 4ml of 80% EtOH, chromatographed on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) (3X40cm) and then eluted with 80% EtOH at flow rate 1ml.min⁻¹ (controlled by a peristaltic pump) to obtain a series of

fractions (4ml each). The fractions obtained were monitored using TLC-Silica gel in solvent system-III and compounds were visualized by spraying with 10% H_2SO_4 in MeOH followed by heating at 100°C for 10min. Flavonoid-1 was found in fractions no 55-63. These fractions were combined and evaporated under reduced pressure at 40°C then redissolved in 1.0ml water followed by freezing and freeze drying to obtain 7mg flavonoid-1.

2.4.2.2. Isolation of flavonoids-2 & 3

Flavonoids-2 and 3 were isolated from the aqueous fraction. A portion of extract (6g) was dissolved in 16ml of 80% EtOH and 4.0ml was applied to Sephadex LH-20 at a time as for EtOAc fraction. Fractions 51-62 contained mainly two flavonoids. These fractions were combined and evaporated and redissolved in MeOH then separated using preparative TLC in solvent system-III. Flavonoid-2 (R_f 0.45) and flavonoid-3 (R_f 0.28) were re-eluted with MeOH and evaporated under reduced pressure at 40°C . Dry flavonoids were redissolved in 1.0ml water then frozen and freeze dried to obtain 3mg of flavonoid-2 and 2mg flavonoid-3.

2.4.3. Isolation of an unknown blue spot phenolic

Fractions no 40-48 of Sephadex LH-20 column chromatography of the aqueous extract contained a blue spot (R_f 0.47) on their TLC chromatograms after spraying with 10% H_2SO_4 in MeOH. This compound was isolated by preparative TLC using solvent system-III.

2.4.4. Isolation of conjugated hydroxycinnamates

Sephadex LH-20 column chromatography combined with preparative TLC have been used to isolate conjugated hydroxycinnamates in the aqueous extract. In both chromatographic systems the compounds were poorly separated, unstable and bound strongly by the silica gel, hence the result was unsatisfactory. A small scale isolation of radioactively labelled compounds was carried out using analytical HPLC and the eluted compounds were collected using a fraction collector.

2.4.5. Identification of isolated compounds

Flavonoids were identified using UV-spectrophotometry with diagnostic shift reagents (Mabry, 1969). The reagents used were a solution of 2.5g metallic sodium in 100ml MeOH, 5% AlCl₃ in MeOH, 4N HCl, NaOAc powder, boric acid powder and a saturated solution of boric acid in MeOH. The acid hydrolysis was carried out by refluxing 1.0mg of flavonoid in 1.0ml of 2N HCl at 100°C (water bath) for 2h. The free flavonoid released was extracted 3X with 1.0ml EtOAc-Et₂O (1:1) and co-chromatographed on HPLC with an authentic marker. The sugar moiety was identified using a Dionex HPLC chromatograph with a pulse amperometric detector, a CarboPac^{PA1} Dionex column, solvent system from 0min. to 10min. with a gradient of 90% H₂O/10% 100mM NaOH to 80% H₂O/20% 100mM NaOH then isocratic elution from 10min. to 30min. and a flow rate at 1ml.min⁻¹. The proton NMR spectra of flavonoids-1 and 2 were also measured. Due to inadequate amounts of flavonoids isolated no significant peak was observed.

Labelled isolates of conjugated hydroxycinnamates were hydrolyzed in acid (2N HCl, 100°C for 2h.), in alkali (0.5N NaOH, room temperature for 24h.) and in a solution of emulsin (β -glucosidase from almond) at pH 5, 30°C, for 24h. The hydroxycinnamates released were identified using TLC-RITA. The sugars were identified by TLC-cellulose using solvent systems-IV and V.

2.5. Synthesis of Putative Capsaicin Precursors

2.5.1. Synthesis of [U-¹⁴C] Cinnamic acid

The method for preparing [U-¹⁴C] cinnamic acid was as described by Jeff *et al.* (1978), using [U-¹⁴C] phenylalanine as starting material. This involved one major operation with possibly two reaction steps (see Fig. 2.5.1). Using this procedure [U-¹⁴C] cinnamic acid with a high specific activity can be achieved.

2.5.1.1. Preliminary experiment using cold phenylalanine

To 1.0mg phenylalanine 2.0ml of 3N NaOH was added. The solution was stirred using a magnetic stirrer and 0.1ml dimethylsulphate slowly added, the mixture was then left for 45min. at room temperature before the addition of a further 0.1ml dimethylsulphate and left for another 45min. The reaction mixture was transferred into a 100ml round bottom flask and evaporated to dryness under reduced pressure at 60°C. The residue was then dissolved in 25ml water, acidified with 6N HCl to pH 3 and extracted 3X with equal volume of 25ml EtOAc-Et₂O (1:1). Combined organic fractions were dried with anhydrous sodium sulphate and evaporated under reduced pressure. The residue was dissolved in 1.0ml HPLC grade MeOH, filtered, then run on HPLC. The HPLC chromatogram of this reaction product is shown in Fig.2.5.2. Apart from ^{trans}-cinnamic acid, there was a small peak of an unknown compound (peak 1, Rt 13.3). The efficiency of this conversion was 61%.

2.5.1.2. Synthesis using [U-¹⁴C] phenylalanine

As the chemical conversion had not been carried out using a trace amount of starting material, 250µg cold phenylalanine was added into a 1.0ml solution of 50µCi [U-¹⁴C]-phenylalanine (SA 450 mCi.mmol⁻¹) (Amersham, U.K.). Into this solution 1.0ml of 6N NaOH was added and then processed as in the preliminary experiment (.2.5.1.1). The [U-¹⁴C] cinnamic acid produced was purified by preparative TLC-Silica gel using solvent system-I and the chromatogram scanned on RITA (see Fig.2.5.3). The band of [U-¹⁴C] cinnamic acid was scraped and eluted with MeOH. The eluate was evaporated under reduced pressure at 40°C and the final product dissolved in 0.05M phosphate buffer at pH 7. The specific activity of [U-¹⁴C] cinnamic acid produced was 24.5mCi.mmol⁻¹ and the efficiency of conversion was 67%. High specific activity of [U-¹⁴C] cinnamic acid was also prepared without the addition of cold phenylalanine without any significant loss in efficiency.

Fig. 2.5.1.

Reaction scheme of the synthesis of cinnamic acid (3) from phenylalanine (1) with trimethyl-N-phenylalanine (2) as a possible intermediate.

Fig. 2.5.2.

HPLC chromatogram of cinnamic acid synthesized from phenylalanine according to the method of Jeffs (1978), 1: unknown compound, 2: cinnamic acid, HPLC system was as described in 2.3.4.
trans-

Fig. 2.5.1.

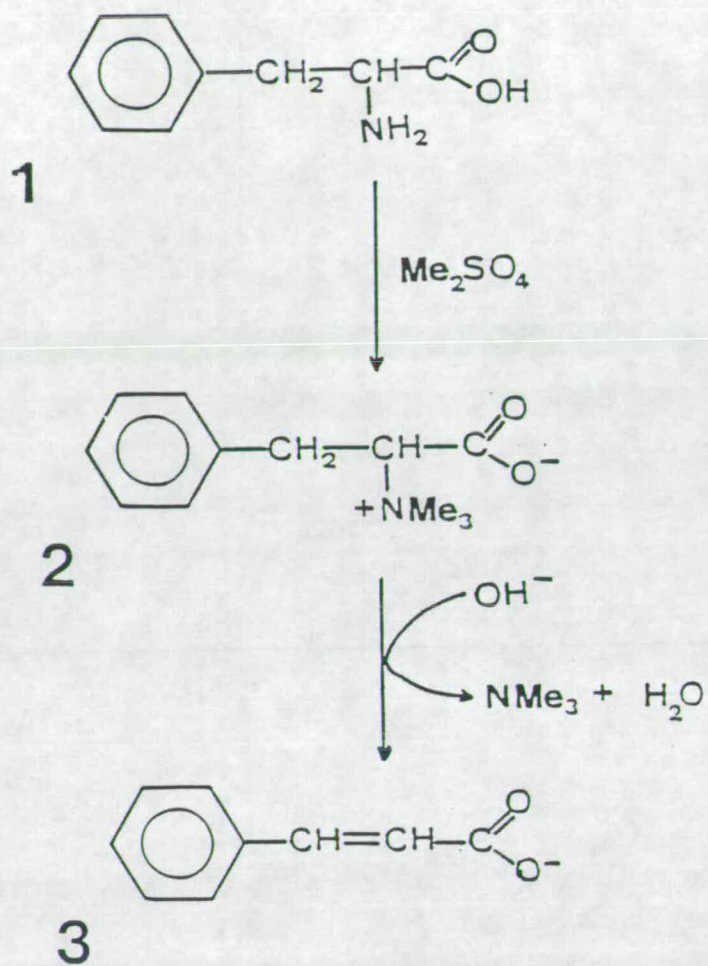
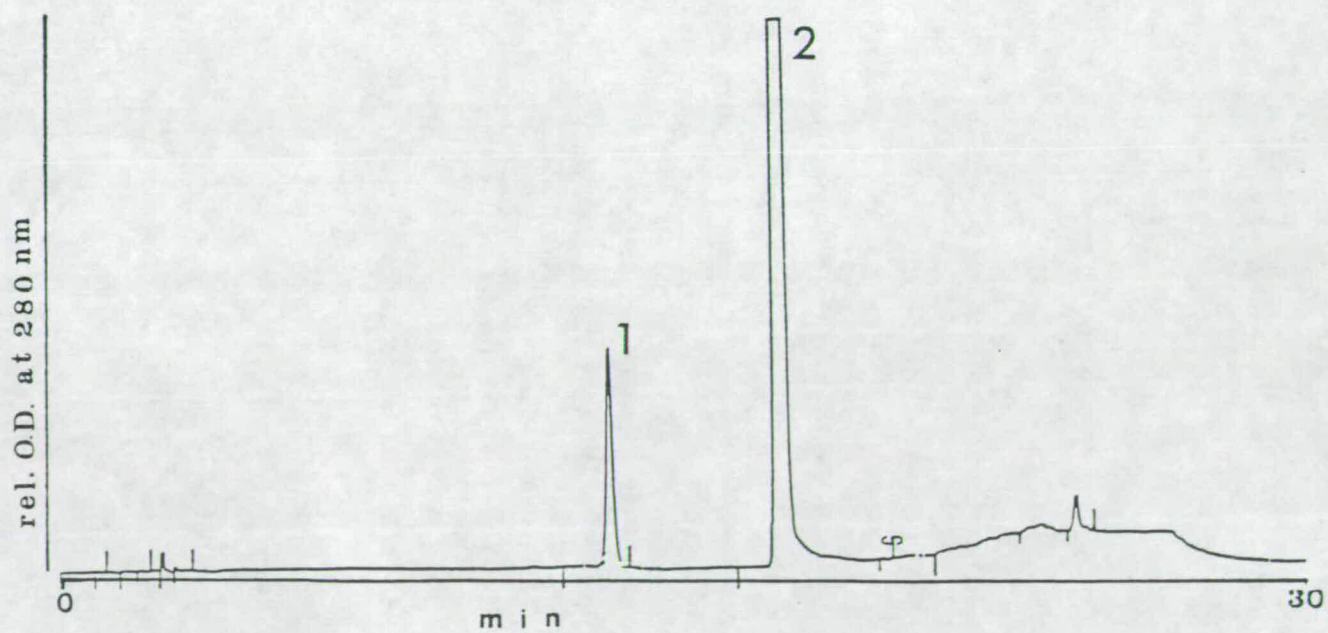


Fig. 2.5.2.



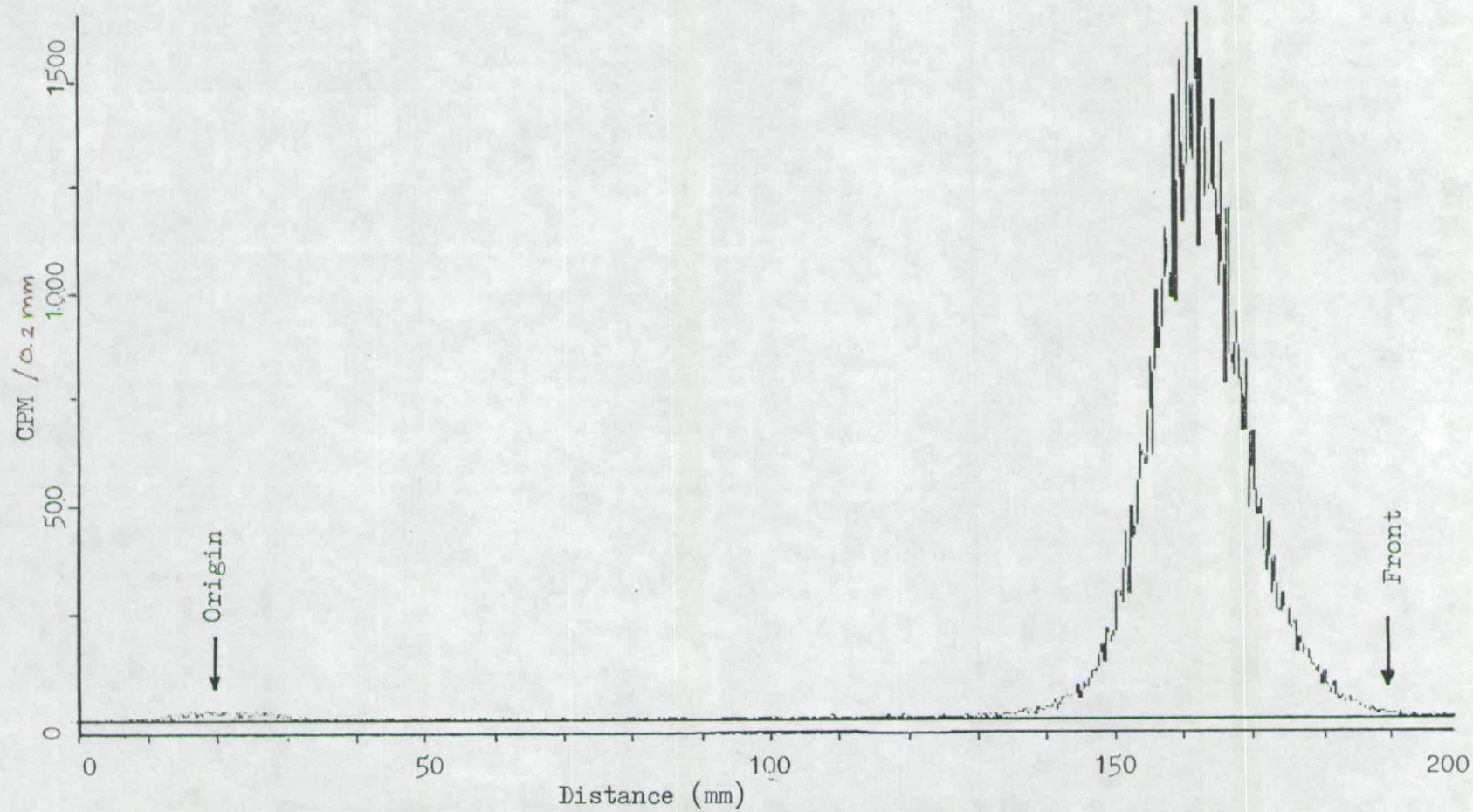


Fig. 2.5.3. TLC-RITA scan of [U- ^{14}C] cinnamic acid synthesized from [U- ^{14}C] phenylalanine according to the method of Jeff *et al.* (1978). TLC on Silica gel-K5 (Whatman) using solvent system-I.

2.5.2. Synthesis of [2-side chain- ^{14}C] p-coumaric acid

p-Coumaric acid labelled at the 2-side chain position can be synthesized using the Knoevenagel reaction with p-OH-benzaldehyde and [2- ^{14}C] malonic acid as starting materials (Austin & Meyers, 1965; Kirk & Brunow, 1988). The reaction scheme is presented in Fig. 2.5.4. Prior to the synthesis of radioactive p-coumaric acid, a small scale preparation using p-OH-benzaldehyde and malonic acid was carried out. The reaction mixture which contained 1.22mg (10 μmol) p-OH-benzaldehyde, 1.04mg (10 μmol) malonic acid, 1.0ml pyridine and 10 μl piperidine was refluxed at 60°C for 24h. The reaction mixture was diluted with 5.0ml water, acidified with 6N HCl to pH 3 and extracted 3X with equal volumes of 5.0ml EtOAc-Et₂O (1:1). Combined organic fractions were evaporated and the p-coumaric acid produced was analysed by TLC and UV spectrophotometry. The efficiency of this reaction was 70%.

Using the same procedure [2-side chain- ^{14}C] p-coumaric acid was synthesized using 60 μCi (24 μmol) [2- ^{14}C] malonic acid (2.5mCi.mmol⁻¹), 3.5mg p-OH-benzaldehyde, 1.5ml pyridine and 15 μl piperidine. The [2-side chain- ^{14}C] p-coumaric acid produced was purified by preparative TLC-Silica gel using CHCl₃-EtOAc-HOAc (70:30:2) as the mobile phase. The chromatogram was scanned on RITA (see Fig. 2.5.5). The band of [2-side chain- ^{14}C] p-coumaric acid was treated as the [U- ^{14}C] cinnamic acid in 2.5.1.2. Finally the [2-side chain- ^{14}C] p-coumaric acid was dissolved in 0.8ml of 0.05M phosphate buffer at pH 7. The total activity obtained was 36.5 μCi (61% yield).

2.5.3. Synthesis of [2-side chain- ^{14}C] caffeic acid

The method of synthesis for [2-side chain- ^{14}C] p-coumaric acid cannot be directly applied for the preparation of [2-side chain- ^{14}C] caffeic acid, as both starting material and final product are unstable under the reaction conditions. To minimize oxidation, catechol was added and the reaction mixture kept in the dark, under nitrogen, at room temperature for one week.

To prepare [2-side chain- ^{14}C] caffeic acid, the reaction mixture contained 40 μCi

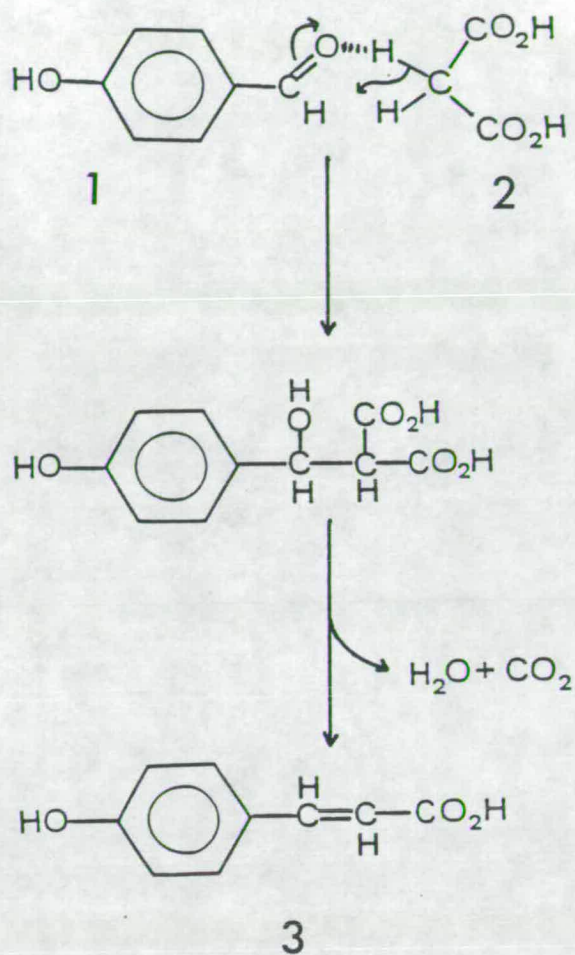


Fig. 2.5.4. Reaction scheme for the synthesis of p-coumaric acid (3) from p-hydroxy benzaldehyde (1) and malonic acid (2).

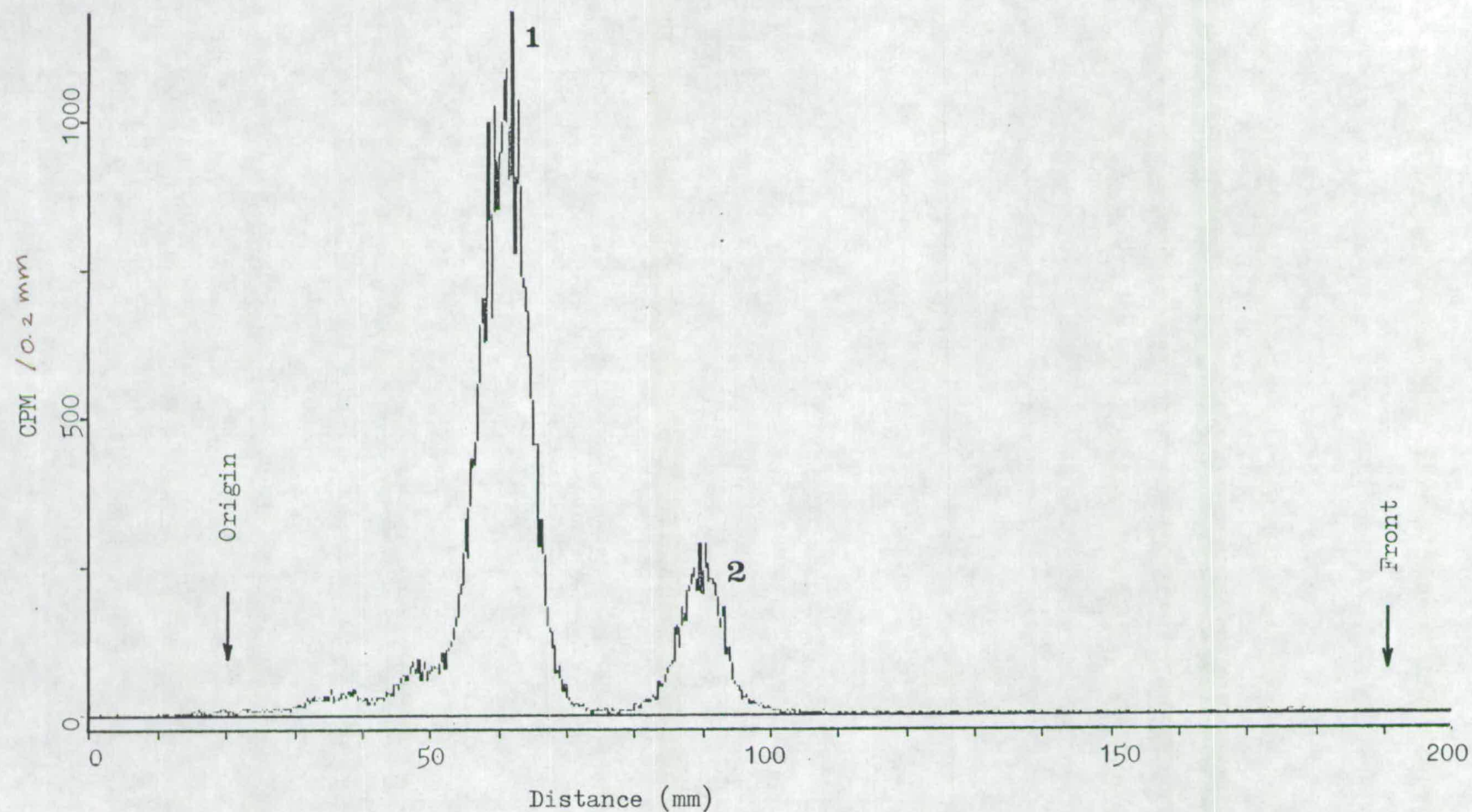


Fig. 2.5.5. TLC-RITA scan of the reaction product between [2- ^{14}C] malonic acid and p-OH-benzaldehyde, 1: p-coumaric acid and 2: unknown compound. TLC on Silica gel plate with $\text{CHCl}_3\text{-EtOAc-HOAc}$ (70:30:2) as solvent.

(16 μmol) [2- ^{14}C] malonic acid (2.5mCi.mmol $^{-1}$ (Sigma), 3.1mg (22 μmol) protocatechuic aldehyde, 10mg catechol, 1ml pyridine and 10 μl piperidine. The reaction product was extracted as for p-coumaric acid (2.5.2) and purified using preparative TLC-Silica gel with CHCl_3 -EtOAc-HOAc (50:50:2) as the mobile phase. The band of caffeic acid was further processed as for [U- ^{14}C] cinnamic acid in 2.5.1.2. The isolated [2-side chain- ^{14}C] caffeic acid was dissolved in 400 μl 0.05M phosphate buffer at pH 7 and analysed using TLC in solvent system-I followed by RITA scanning (see Fig. 2.5.6). The total activity of [2-side chain- ^{14}C] caffeic acid was 3.4 μCi (8.5% yield).

2.5.4. Synthesis of [3,5- ^3H] p-coumaric acid

A method for synthesis of a high SA ring radioactively labelled p-coumaric acid was developed using PAL (EC 4.3.1.5) from *Rhodotorula glutinis* (Sigma). This enzyme converts phenylalanine to cinnamic acid with a high efficiency, and also has a reduced ability to convert tyrosine to p-coumaric acid.

In a preliminary experiment 1mg tyrosine was incubated with 0.08unit of enzyme in 1.5ml 0.1M Tris buffer (pH 8.8) for 24h. The reaction mixture was then acidified to pH 3 and the product extracted as for p-coumaric acid in 2.5.2. Combined organic fractions were evaporated and redissolved in 1.0ml HPLC grade MeOH and analysed by HPLC (see Fig. 2.5.7). The byproduct a compound of peak-a in Fig. 2.5.7 has been confirmed to be due to a chemical rather than an enzymic reaction. The formation of this compound was reduced by degassing the reaction mixture with helium, shortening the incubation period or by the addition of ferulic acid. This compound was identified as *cis* p-coumaric acid by comparing its R_t value and UV spectrum with authentic p-coumaric acid (Sigma) which contains both *cis* and *trans* isomers. *Cis* p-coumaric acid production was further inhibited when the reaction volume was reduced to 250 μl and a buffer at pH 8.0 was used. This procedure was finally employed to synthesize [3,5- ^3H] p-coumaric acid from [3,5- ^3H] tyrosine (SA 51Ci.mmol $^{-1}$) (Amersham, U.K.). The reaction mixture contained 100 μCi tyrosine (100 μl), 0.08unit of enzyme (40 μl) and 100 μl 0.05M Tris buffer at pH 8. After 12h.

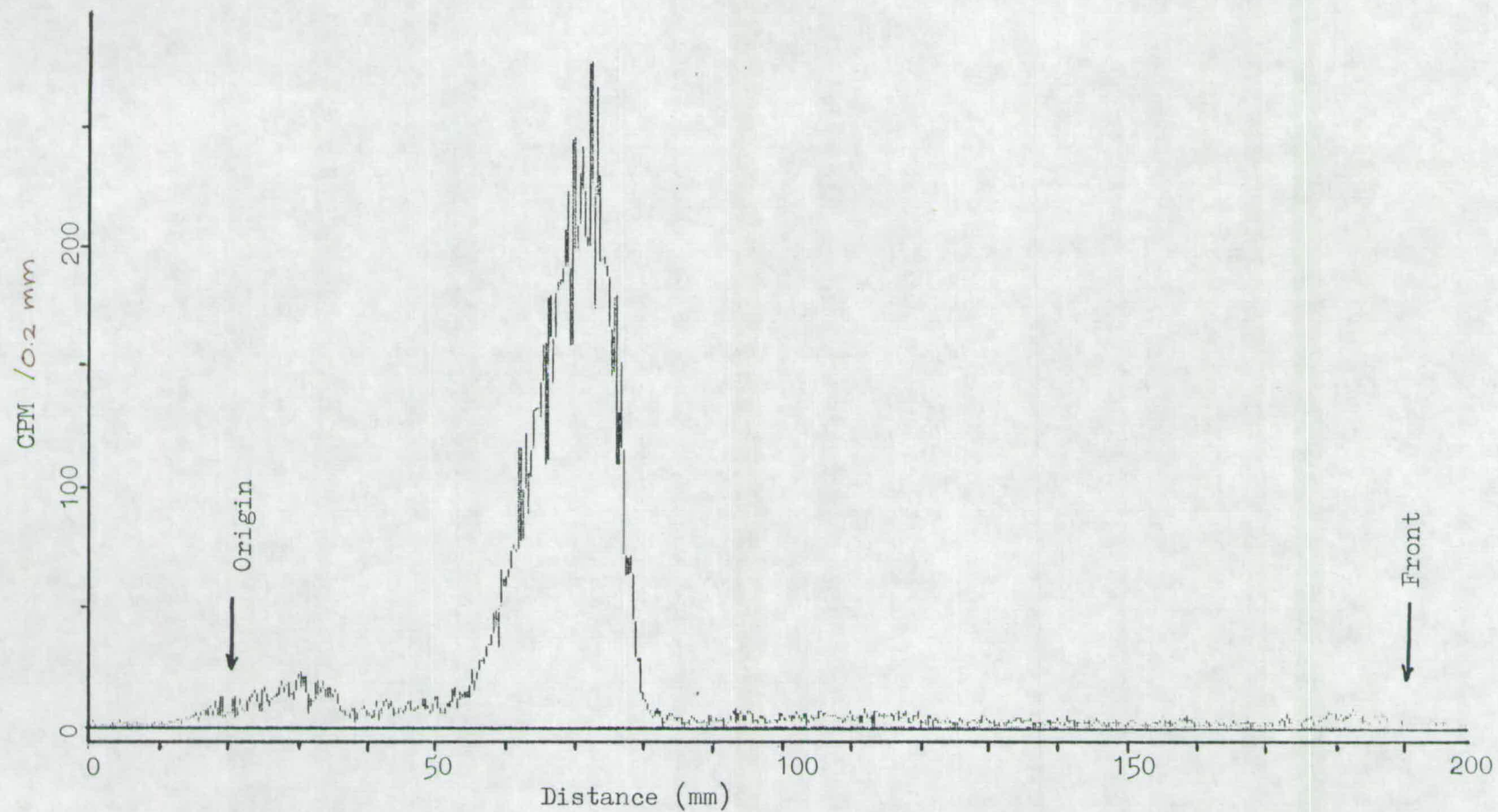


Fig. 2.5.6. TLC-RITA scan of [2-side chain- ^{14}C] caffeic acid synthesized from protocatechuic aldehyde and [2- ^{14}C] malonic acid. TLC on Silica gel (E Merck) using solvent system-I.

Fig. 2.5.7.

HPLC chromatograms of the reaction product of tyrosine (1mg) incubated with PAL from *Rhodotorula glutinis* (Sigma) in 0.05M Tris buffer (pH 8.8), at 30°C for 24h. A: 2D-plot, B: 3D-plot, a: ^{cis}~~trans~~ p-coumaric acid and b: ^{trans}~~cis~~ p-coumaric acid. HPLC system was as described in 2.3.4 with a slight modification to the solvent gradient and time of elution.

41

Absorbance [mAU]

2666.6 B

1800

1200

600

222

242

262

282

Wavelength [nm]

302

322

7.7

8.4

9.1

9.8

Time [min]

10.5

11.2

11.9

A
rel. OD at 280nm

0

min

15

a

b

B

incubation, 1.0ml MeOH was added. The solution was then chromatographed on a preparative TLC-Silica gel plate in solvent system-I. The RITA scan of the chromatogram is shown in Fig. 2.5.8. The band of [3,5-³H] p-coumaric acid was further processed as for [U-¹⁴C] cinnamic acid (2.5.1.2). The labelled p-coumaric acid obtained was 79.8μCi (80% yield) and was dissolved in 1.0ml of 0.05M phosphate buffer at pH 7.

2.5.5. Synthesis of [U-¹⁴C] p-coumaric acid

p-Coumaric acid tritiated at position 3 and 5 did not give satisfactory results for tracing the synthesis of capsaicin as one of the tritium atoms is removed from the molecule on hydroxylation to caffeic acid^{and in general, the levels of incorporation into capsaicin are low.} However, it allowed the uptake of p-coumaric acid by the cells to be quantified. This was low at pH 7.0 but higher at pH 5.5. In a more detailed study of the fate of p-coumaric acid in *Capsicum frutescens* fruits [U-¹⁴C]-p-coumaric acid was used. This compound was synthesized from [U-¹⁴C]-tyrosine (SA 460mCi.mmol⁻¹) (Amersham, U.K.). Since the activity *per* unit volume of [U-¹⁴C] tyrosine (50μCi.ml⁻¹) was much lower than [3,5-³H] tyrosine (1000μCi.ml⁻¹), a slight modification to the method is required. Before the synthesis was carried out, a time course for conversion of tyrosine to p-coumaric acid was studied under the following conditions which were subsequently used to produce [U-¹⁴C] p-coumaric acid. The reaction mixture, which contained 4μg^{of cold} tyrosine (200μl) ^{equivalent to the level of tyrosine in} [equal to] 10μCi of [U-¹⁴C] tyrosine (460mCi.mmol⁻¹), 0.08unit of enzyme (15μl) and 50μl of 0.05M Tris buffer at pH 8, was incubated at 30°C. To facilitate the measurement of small amounts of the starting material and the product, 5μCi [3,5-³H] tyrosine (5μl) was added to the reaction mixture. Ten microlitre samples were taken after 5min., 1h., 3h., then every 3h. until 15h. The results presented in Fig. 2.5.9 show that within 1h. most tyrosine has been converted into p-coumaric acid.

To synthesize [U-¹⁴C] p-coumaric acid, 1.0ml of 50μCi [U-¹⁴C] tyrosine was divided into 5 aliquots of (200μl each) then to each aliquot 0.08unit of enzyme (15μl) and 50μl buffer was added. The reaction mixtures were incubated at 30°C for 3h. and

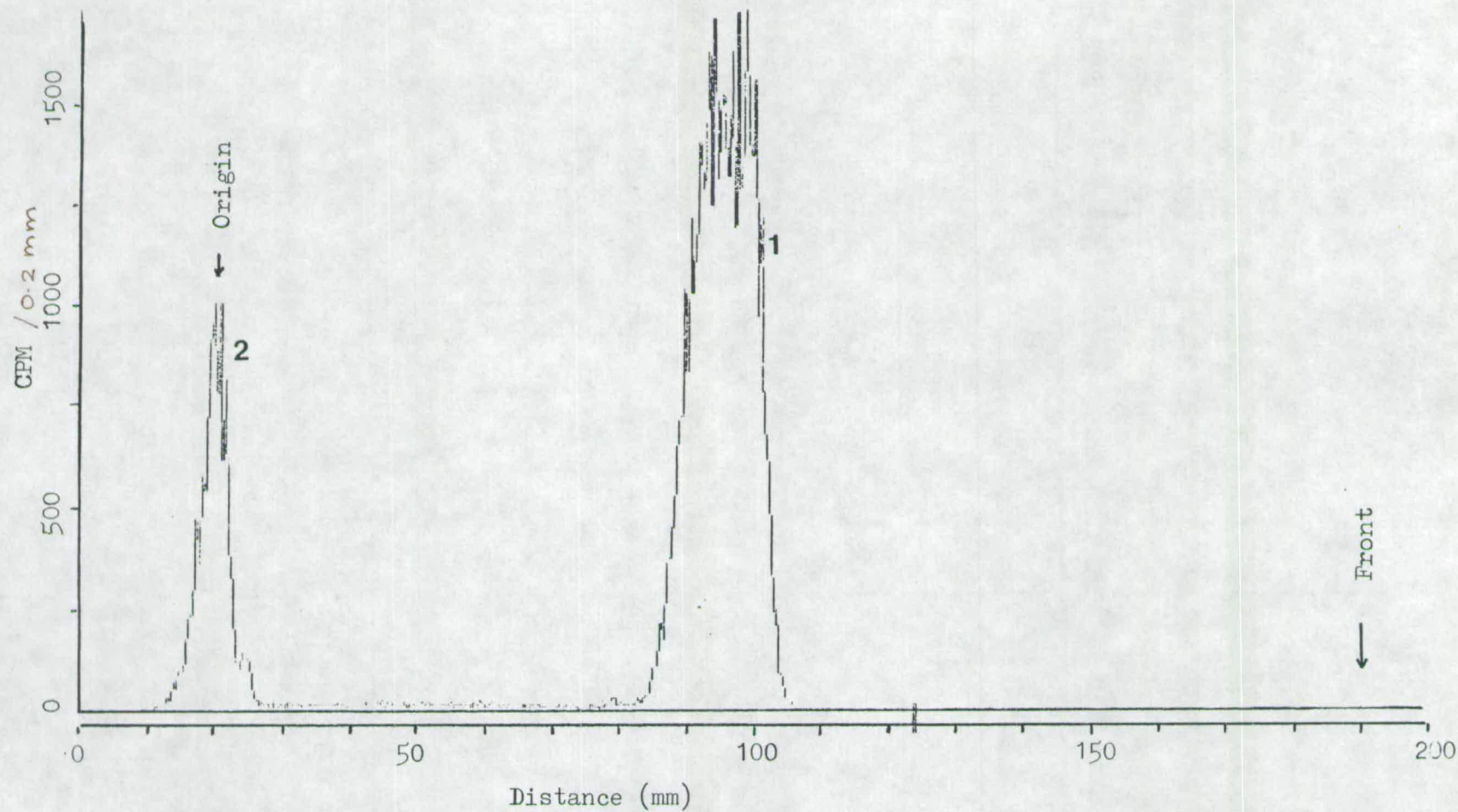


Fig. 2.5.8. TLC-RITA scan of the reaction product of [3,5- ^3H] Tyrosine (Amersham, U.K.) incubated with PAL from *Rhodotorula glutinis* (Sigma) in 0.025M Tris buffer (pH 8) at 30°C for 12h, 1: p-coumaric acid, 2: tyrosine. TLC on Silica gel K5 (Whatman) with solvent system-I.

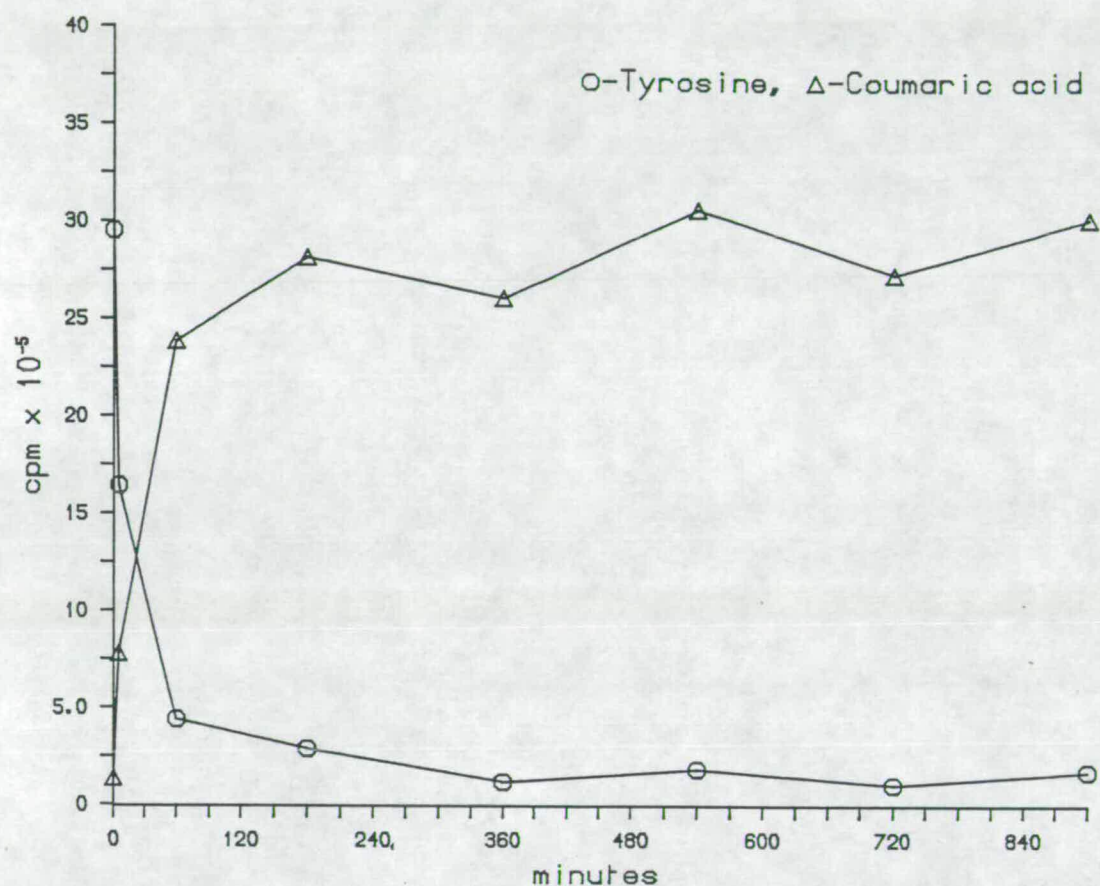


Fig. 2.5.9. Time course of the conversion of tyrosine to p-coumaric acid under the conditions designated for the synthesis of [U-¹⁴C] p-coumaric acid from [U-¹⁴C] tyrosine (Amersham, U.K.).

the reaction stopped by the addition of 1.0ml MeOH to each tube. The solution was then transferred to a 100ml round bottom flask and evaporated under reduced pressure at 40°C. The residue was then chromatographed on a preparative TLC-Silica gel plate in solvent system-I. The band of [U-¹⁴C] p-coumaric acid was further processed as for [U-¹⁴C] cinnamic acid 2.5.1.2. The [U-¹⁴C] p-coumaric acid obtained was 47μCi (94% yield) and dissolved in 1.0ml of 0.01M phosphate buffer at pH 5.5.

2.5.6. Synthesis of p-Coumaroyl-β-D-glucose

The glucosylation of p-coumaric acid involves protection of the hydroxy group, activation of the acyl group, conjugation with tetraacetyl-β-D-glucose and removal of the protecting groups. The scheme of these reactions is summarized in Fig.2.5.10.

2.5.6.1. Acetylation of p-coumaric acid

The hydroxy group of p-coumaric acid was protected by acetylation as described by Shimizu & Kojima (1984). p-Coumaric acid (3g=18mmol) was dissolved in 15ml pyridine and 3g acetic anhydride (29mmol). The mixture was left for 24h. at room temperature. The reaction mixture was then poured into 100ml ice water and crystallization was left to proceed overnight. The white crystals produced were filtered and dried using Büchner funnel fitted with Whatman No.1 filter paper (Whatman). The yield of p-acetylcoumaric acid obtained was 2.25g (10.8mmol).

2.5.6.2. Preparation of p-acetylcoumaroylchloride

For esterification, p-acetylcoumaric acid was first activated by forming an acylhalide (acylchloride). The method for the synthesis of p-acetylcoumaroylchloride was as described by Shimizu & Kojima (1984). p-Acetylcoumaric acid (2.25g) was refluxed with 15ml thionylchloride in a water bath at 100°C for 3h., the excess thionylchloride removed by evaporation under reduced pressure at 60°C to leave a yellow liquid. This liquid crystallized on cooling and the p-acetylcoumaroylchloride produced (2.30g) was used for the next step of the synthesis.

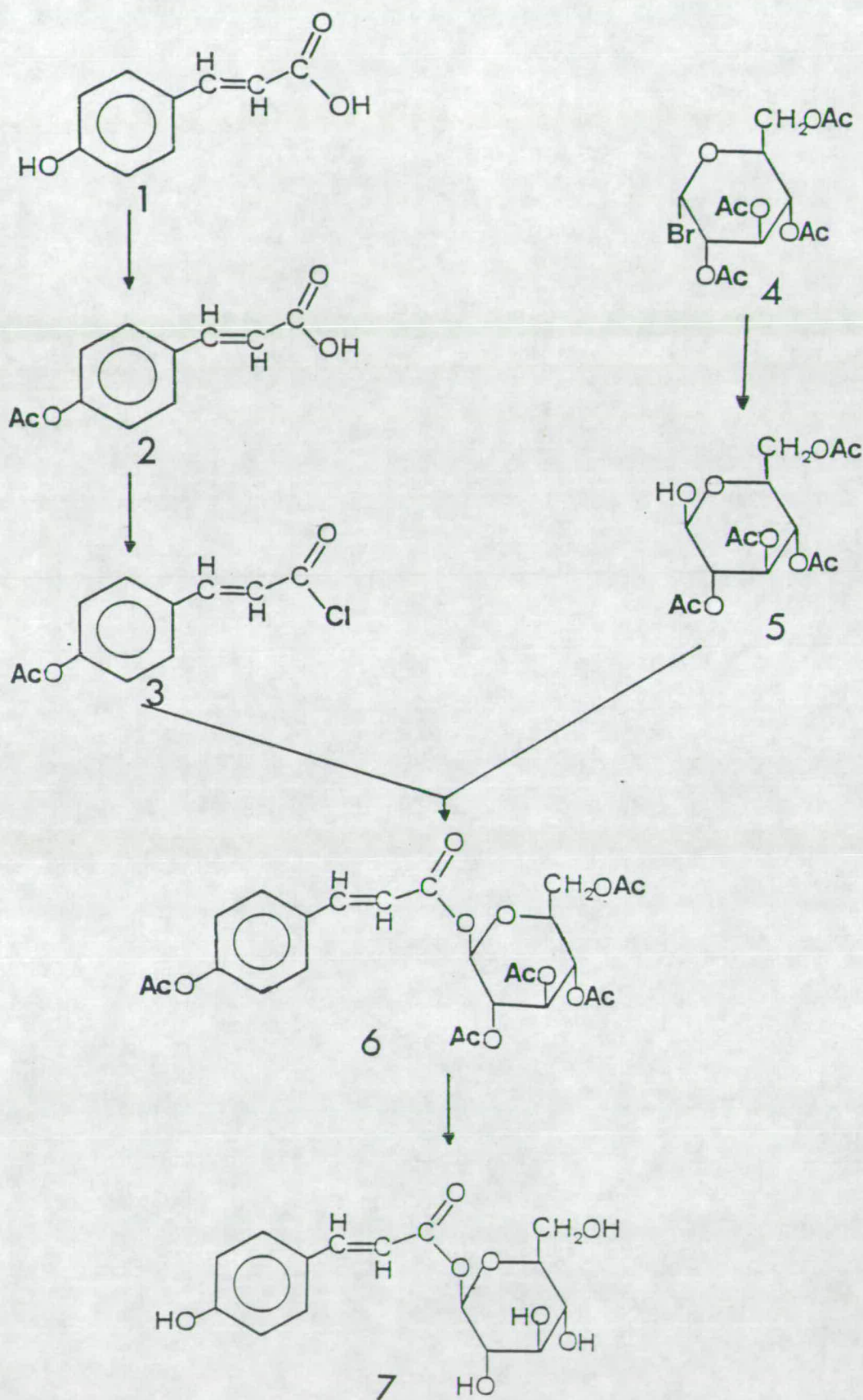


Fig. 2.5.10. The reaction scheme for p-coumaroyl-β-D-glucose synthesis, 1. p-coumaric acid, 2: p-acetylcoumaric acid, 3: p-acetylcoumaroylchloride, 4: acetobromo-α-glucose, 5: tetraacetyl-β-D- glucose, 6: acetylated p-coumaroyl-β-D-glucose, 7: p-coumaroyl-β-D-glucose.

2.5.6.3. Preparation of tetraacetyl- β -D-glucose

Tetraacetyl- β -D-glucose was prepared from 2,3,4,6-tetraacetyl- α -D-glucopyranosylbromide (acetobromo- α -D-glucose) (Sigma) and silver carbonate (McCloskey & Coleman, 1955). The latter was freshly prepared by the dropwise addition of a sodium carbonate solution (1.2g in 10ml water) into a silver nitrate solution (3.5g in 40ml water) whilst stirring. The yellow precipitate of silver carbonate was filtered through a Buchner funnel fitted with Whatman No.1 filter paper (Whatman) and washed with acetone to facilitate drying. To avoid reduction of the silver ion, all operations were carried out in the dark. Dry silver carbonate (2.8g) was ground and used directly for the preparation of tetraacetyl- β -D-glucose.

A solution of acetobromo- α -D-glucose (5g in 10ml dry acetone) was cooled in an ice bath at 0°C and 200 μ l water added. While stirring, 2.8g of silver carbonate powder was added slowly to the solution and stirring was continued for a further 30min. The silver bromide crystals were filtered through a Buchner funnel fitted with Whatman No.1 filter paper (Whatman) and washed with acetone. Washing was repeated by warming the silver bromide in acetone followed by filtration. Combined filtrates were evaporated under reduced pressure at room temperature to obtain a white powder of 2,3,4,6-tetraacetyl - D-glucose (3.50g).

2.5.6.4. Synthesis of p-acetylcoumaroyl-2,3,4,6-tetraacetyl - D-glucose

The method for conjugation of p-acetylcoumaroylchloride with 2,3,4,6-tetraacetyl - D-glucose was as described by Birkofer *et al.* (1961). p-Acetylcoumaroyl chloride (2.30g) was dissolved in 15ml CHCl₃ containing 3.0ml pyridine, then 3.50g 2,3,4,6-tetraacetyl - D-glucose was added. The compounds were dissolved by gently heating, then the solution stirred at room temperature for two days. Into the reaction mixture 25ml CHCl₃ was added and the solution subsequently extracted with equal volumes of 25ml 2N H₂SO₄, 2N NaHCO₃ and water. The CHCl₃ fraction, which

contained the p-acetylcoumaroyl,2,3,4,6 - D-glucose, was evaporated under reduced pressure at 30°C.

2.5.6.5. Deacetylation of acetylated p-coumaroyl - D-glucose

Acetyl protecting groups were removed from the ester glucoside according to the method of Birkofer *et al.*(1966). Acetylated p-coumaroyl - D-glucose (2.1g) was dissolved in 20ml MeOH and cooled to 0°C in an ice bath. During this process, white crystals were observed. To this suspension, 20ml of 0.1N NaOMe (sodium methoxide) was added and the solution stirred for 60min. Amberlite-IR120 (cation exchanger) (Sigma) was added as necessary whilst stirring to neutralize the solution. The mixture was then filtered and the filtrate was evaporated under reduced pressure. The residue was suspended in 25ml water and extracted three times with equal volumes of 25ml EtOAc-Et₂O (1:1). The water fraction was evaporated to dryness under reduced pressure at 60°C and the residue dissolved in a small volume of MeOH. p-Coumaroyl - D-glucose was recrystallized by adding EtOAc-hexane (1:1) dropwise until the solution became cloudy. After storing at -20°C overnight the crystals formed were filtered off under reduced pressure, washed with EtOAc-hexane (1:1) and dried. The yield obtained was 155mg.

2.5.6.6. Identification of p-coumaroyl-β-D-glucose

A solution of 1mg.ml⁻¹ was prepared for HPLC analysis. The chromatogram (Fig.2.5.11.a) shows that the major peak constituted $\sqrt{\hspace{1cm}}$ The UV spectrum was measured in MeOH. Fig.2.5.11.b. shows the red shift of the λ_{max} after the addition of three drops of N NaOH which indicates the presence of free OH phenolic. On incubation with 1mg emulsin (Sigma) in 0.05M acetate buffer (pH 5) at 30°C for 24h, the compound released p-coumaric acid which shows that the p-coumaric acid was β-glucosylated. Alkaline hydrolysis of the compound in N NaOH for 60min followed by demineralization with Amberlite-IR120 (cation exchanger) released glucose (Fig.2.5.11.c). The above results suggested the compound was p-coumaric acid esterified with β-D-glucose.

$\sqrt{\hspace{1cm}}$ approximately 96% of the total UV absorbing materials at 270nm in the product.

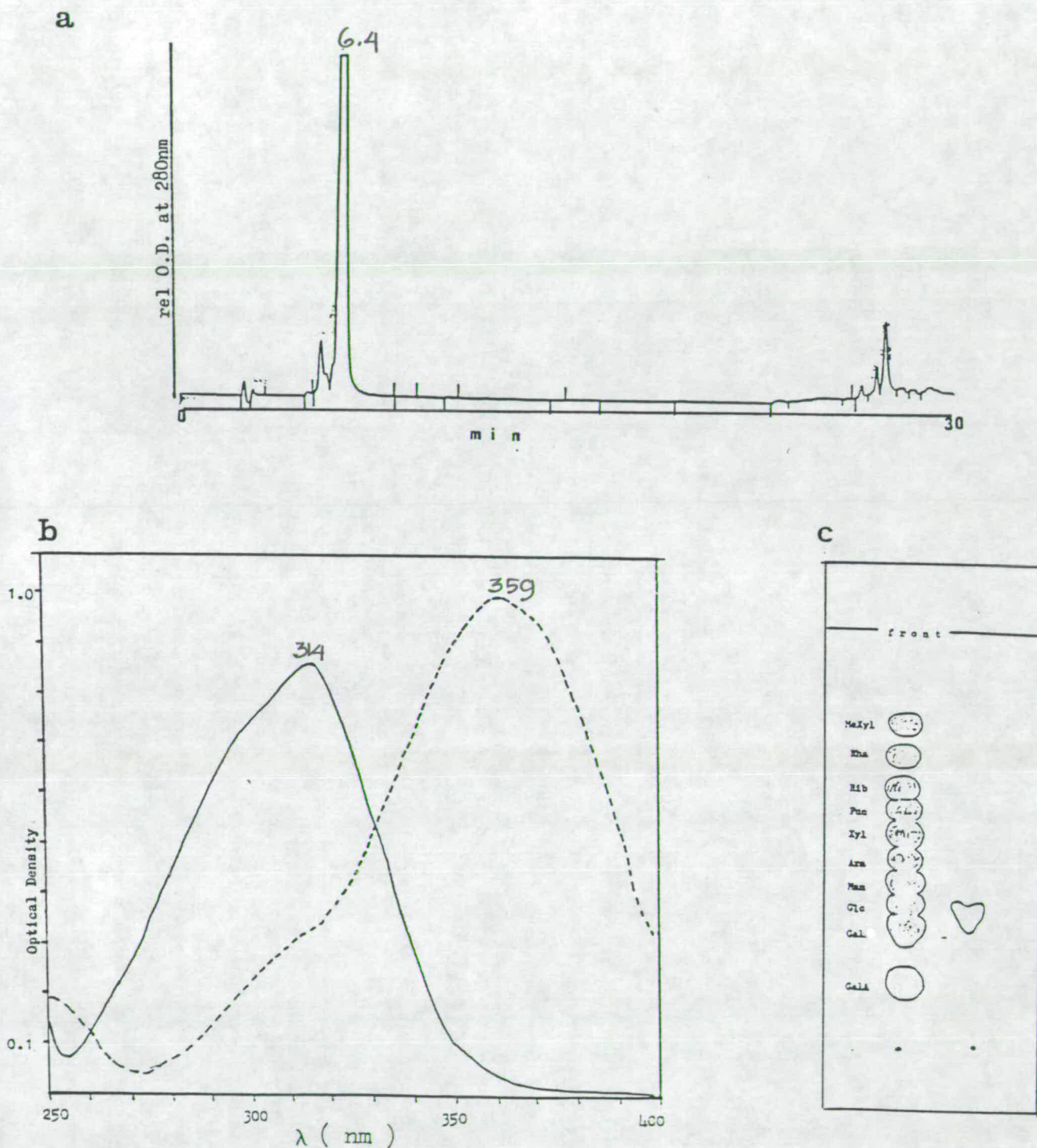


Fig. 2.5.11. Identification of the synthesized coumaroyl- β -D-glucose. a. HPLC chromatogram, b: UV spectra: — in MeOH, --- in MeOH + 3drops of N NaOH, c: TLC chromatogram of sugar (S) released on alkaline hydrolysis and M is a mixture of 10 common monosaccharides. HPLC system was as described in 2.4.3 with modifications to the solvent gradient. TLC on cellulose (E.Merck) using solvent system-IV and V.

2.6. Extraction and Assay of β -Glucosidase Activity in Fruits

2.6.1. Preparation of crude enzyme extract

The crude enzyme extract was prepared according to the method of Surholt & Hoesel (1978). All operations in this experiment were carried out at 0-5°C, unless otherwise stated. Fruits (at least 10), PVP-40T (MW 40,000) and 0.05M acetate buffer (pH 5) containing 10 μ M mercaptoethanol (1:0.025:1.5, w/w/v) were ground in a mortar with quartz sand. The homogenate was squeezed through a double layer of muslin cloth and the final volume of filtrate adjusted to 2X fruit FW (v/w) using buffer to rinse the cell residue. Filtrate was centrifuged at 2000Xg for 20min. Glycosylated phenolics which may still be in the crude enzyme extract were removed by eluting the extract through a prepacked column-PD-10 Sephadex G25 (Pharmacia, Uppsala, Sweden) (Holland, 1989). The column was equilibrated with 25ml buffer and then 3.0ml of supernatant was applied and the eluate collected. The column was rinsed with 3.0ml buffer to give a total extract volume of 6.0ml.

2.6.2. Estimation of soluble protein content in the enzyme extract

Protein contents of the enzyme extracts were determined colorimetrically using the method of Bradford (1976). This method is based on the colour produced by a complex formation between protein and a dye (Coomassie Brilliant Blue). In acid this dye has two forms with different colours, blue and orange. Proteins preferentially produce a complex with the blue form with an extinction coefficient at 595nm much higher than the free dye. The dye solution was prepared by dissolving 50mg Coomassie Brilliant Blue (Sigma) in 25ml EtOH followed by addition of 50ml phosphoric acid and water to a final volume of 500ml. The solution was then filtered through Whatman No.1 filter paper.

Protein samples were obtained by adding a 20 μ l Trichloroacetic acid(TCA) solution (100% w/v) into 0.5ml of a crude enzyme solution. Precipitation of protein

was left to proceed overnight at 5°C. Precipitates were centrifuged at 10,000Xg for 5min. in an Eppendorf centrifuge. Supernatants were discarded and the pellets washed with 1.0ml TCA (10% w/v) followed by centrifugation at 10,000Xg for 5min. and removal of the supernatants. The pellet was resuspended in 1.0ml of 0.1N NaOH and solubilisation was left to proceed for at least 30min. at room temperature. Bradford reagent (3.0ml) was added to 100µl protein extract and the absorbance measured at 595nm. The amount of protein present was estimated from a calibration curve of Bovine Serum Albumin (BSA) (Sigma) solution which contained 0-100µg protein (Fig. 2.6.1). Dilution of protein samples was carried out, if necessary, to ensure the samples lay on the linear region of the calibration curve.

2.6.3. Determination of the specific activity of β -glucosidase

Most hydroxycinnamic acids in chilli pepper fruits are glycosylated. It has been shown that glucosidases in plants possess a specificity towards the aglycone moiety of the glycoside (Hosel & Totenhagen, 1980; Hosel, 1981). In this experiment synthetic p-coumaroyl- β -D-glucose which can be synthesized in a substantial amount, was used as a substrate.

The assay conditions were as described by Hestrin *et al.* (1955) and Lee (1972). The assay mixture contained 0.5ml enzyme extract, 0.2ml (5µmol) p-coumaroylglucoside and 0.3ml acetate buffer 0.05M pH 5. The same volume of enzyme extract was boiled for 20min. and used as a control. After incubation at 30°C for 2h., the reaction was stopped by the addition of 3 drops 6N HCl, then 1.0ml of water, followed by extraction of the p-coumaric acid as described in 2.5.2. It was impossible to separate p-coumaric acid from its glucoside simply by liquid liquid extraction. Therefore the p-coumaric acid produced was further separated from its glucoside and quantified using HPLC. The HPLC chromatogram of this assay is shown in Fig. 2.6.2. Total enzyme activity (in µkat) was calculated from the p-coumaric acid produced (in µmol) divided by the period of incubation (in sec). The specific activity was obtained by dividing the activity with protein content.



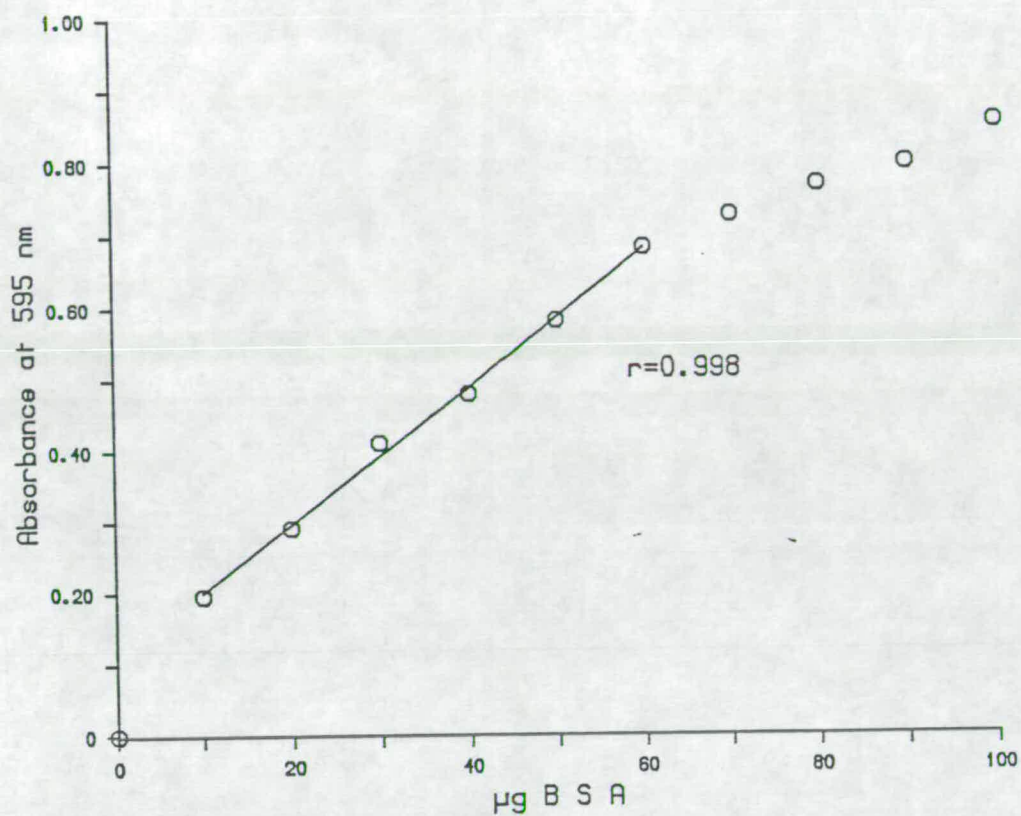


Fig. 2.6.1. Calibration curve for the estimation of protein using BSA as standard (Bradford, 1976)

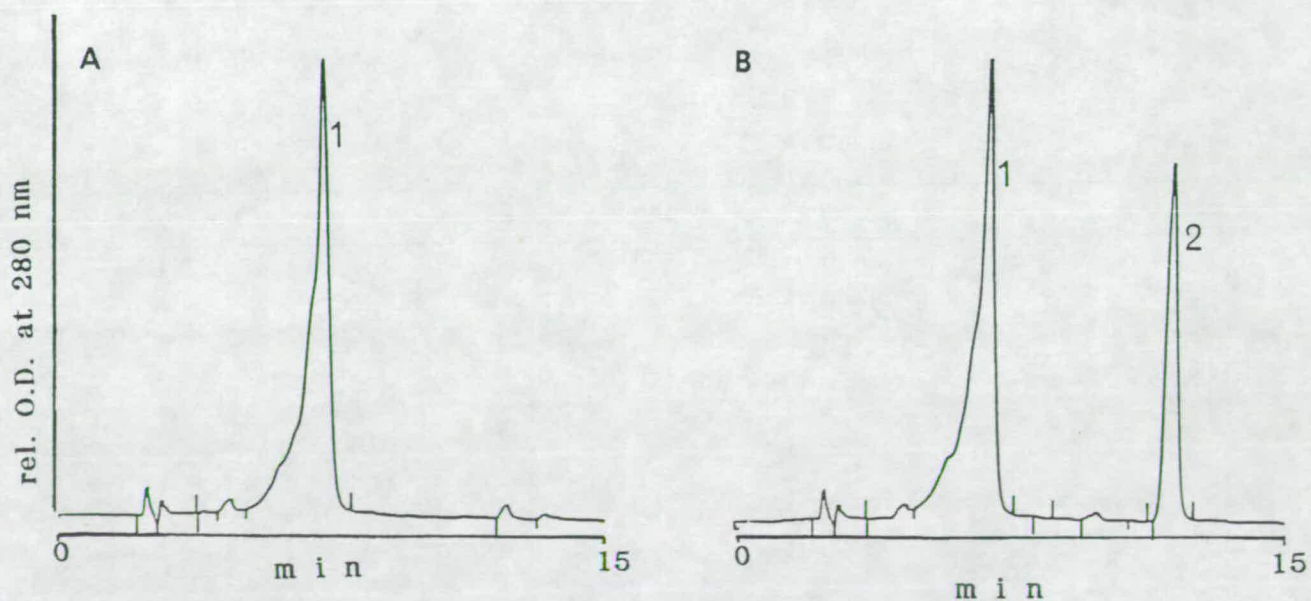


Fig. 2.6.2. Chromatogram of a β -glucosidase assay, A: control, B: assay, 1: p-coumaroyl- β -D-glucose and 2: p-coumaric acid

2.7. Statistical Calculation

In most experiments, at least three replicates were used for each treatment. The standard error of the mean (SE) was calculated to evaluate variation within a treatment and the differences between treatments. Regression analysis was performed to determine the correlation between two variables by calculating the regression equation and correlation coefficient. Calculation of SE, the regression equation and correlation coefficient were as described by Swinscow (1983).

Chapter 3: EXPERIMENTAL RESULTS

Section 3.1. Growth of Cells and Accumulation of Phenolics in Cultures

3.1. Growth of Cells and Accumulation of Phenolics in Cultures

Earlier observations in this laboratory [Holden (unpublished results)] have suggested a possible correlation between the composition of the culture medium and the ability of pepper cells to synthesise and accumulate capsaicinoids. In particular, it was noted that cells grown on MS medium produced higher concentrations of phenylpropanoids than those cultured on SH medium. The differences in the response of the cell cultures to the medium on which they are grown are, at least, in part, related to growth rate. Cells on MS medium grew fairly slowly and form compact aggregates whilst those on SH medium grow rapidly and are dispersed. The aim of these preliminary series of experiments was to study the reported relationship between culture medium and phenylpropanoid metabolism, paying particular attention to the range of free and bound phenolics and other aromatic compounds. This would provide the background information for the major study on capsaicinoid metabolism in cultured cells and fruits of *Capsicum frutescens*.

3.1.1. In Callus Cultures

3.1.1.1. An attempt to identify phenolics in callus cultures

Phenolics in callus cultures of *Capsicum frutescens*, especially water soluble conjugated phenolics, have not so far been investigated. To identify these phenolics in callus, 10g. of fresh 15d. old tissue grown on SH medium was used in each analysis. This tissue was extracted and fractionated as summarized in Fig. 2.3.1. Due to the difficulties encountered in separation and identification of water soluble conjugated phenolics, the phenolic moieties of these compounds were identified after release from their bound form by alkaline, acid and enzymic hydrolysis. Free phenolics and saponifiable cell wall phenolics were also studied.

i. Free phenolics

Free phenolics were present in callus cultures at very low levels and were not easily detectable by TLC and HPLC. The two peaks a and b in Fig. 3.1.2.A in the HPLC chromatogram of the EtOAc-Et₂O fraction are due to the incomplete separation of the EtOAc-Et₂O and aqueous phase during liquid-liquid extraction. This was subsequently confirmed by comparing the HPLC chromatogram of this fraction with non-hydrolysed conjugated phenolics in the aqueous fraction.

ii. Alkaline hydrolysis products of the aqueous fraction

There are significant levels of phenolics bound via ester bonds in callus tissue. Phenolics bound as esters are released from their conjugated form by alkaline hydrolysis, as described in 2.4.5. p-Coumaric, ferulic and vanillic acid were identified by TLC from their colour and location (Fig. 3.1.1.a). These compounds were also detected by HPLC (Fig. 3.1.2.B). Some other unknown phenolics were also detected by both TLC and HPLC.

iii. Acid hydrolysis products of the aqueous fraction

Phenolics conjugated with sugars via O-glycosidic bonds can be released by acid hydrolysis. This hydrolysis, however, will also cleave ester bonds. In this experiment the same sample was used for both alkaline and acid hydrolysis. Following acidification and extraction of free phenolics released by alkaline hydrolysis, the remaining aqueous fraction was hydrolysed with 2N H₂SO₄, by refluxing at 100°C for 2 h. The phenolics released were extracted by liquid-liquid extraction as described in 2.3.2.1 and analysed by TLC and HPLC.

Significant levels of phenolics bound as O-glycosides were present. Vanillic acid, ferulic acid and some unknown phenolics were detected by TLC (Fig. 3.1.1.b). However, on HPLC, these compounds were obscured by large peaks of non-phenolic compounds (peaks 1, 2 and 3 in Fig. 3.1.2.C). These compounds showed a maximum absorbance at 280nm, but did not react with phenolic reagents. The origin of these

compounds was investigated by analysing a crude isolate obtained by passing the MeOH extract of callus through a Bondelut C-18 column (Anachem International) and eluting with water to remove phenolic compounds. To identify this crude isolate, 2mg of material was injected on to the HPLC, but no peak was observed. However, acid hydrolysis of this isolate produced peak-1 as shown on Fig. 3.1.2.C. To determine the chemical nature of this crude isolate 100mg of the isolate, glucose, fructose, mannitol, sucrose and maltose were each hydrolysed in 5ml of 2N H₂SO₄ at 100°C for 2h. The hydrolysis products were then extracted with 3X5ml of EtOAc-Et₂O (1:1) and the organic fractions evaporated under reduced pressure at 40°C. The residues were dissolved in MeOH and their UV spectra measured. From the sugars analysed, only sucrose produced a compound which strongly absorbs at 280nm and has a UV spectrum similar to the hydrolysis product of the crude isolate. Sucrose in the cells may be responsible for the large peaks 1, 2 and 3 on HPLC (Fig. 3.1.2.c) following acid hydrolysis of the MeOH extract.

iv. Emulsin hydrolysis products of the aqueous fraction

Ferulic acid, vanillic acid and some unknown phenolics which were previously found in both the acid and alkaline hydrolysates were released from their conjugated form following enzymic hydrolysis of the aqueous fraction (Fig. 3.1.1.c). Enzymic hydrolysis was carried out by incubating the residue of the aqueous fraction in 5ml of 0.05M acetate buffer (pH 5) containing 1 mg emulsin (β -glucosidase from almond) at 30°C for 24h. This result suggests that most of the phenolics in the water fraction were conjugated with sugars^{provided that emulsin does not contain esterases.} Since alkaline hydrolysis usually breaks the ester bond only, similar to the products released by enzymic hydrolysis product, it is probable that the phenolics in the aqueous fraction were bound with sugars via an ester or O-glycosidic linkage.

v. Saponifiable cell wall phenolics

Saponifiable cell wall phenolics were extracted from the cell residue obtained after continuous extraction of the tissue with MeOH in a Soxhlet apparatus. The extraction of saponifiable cell wall phenolics was as described in 2.3.2.1. Fig. 3.1.2.D

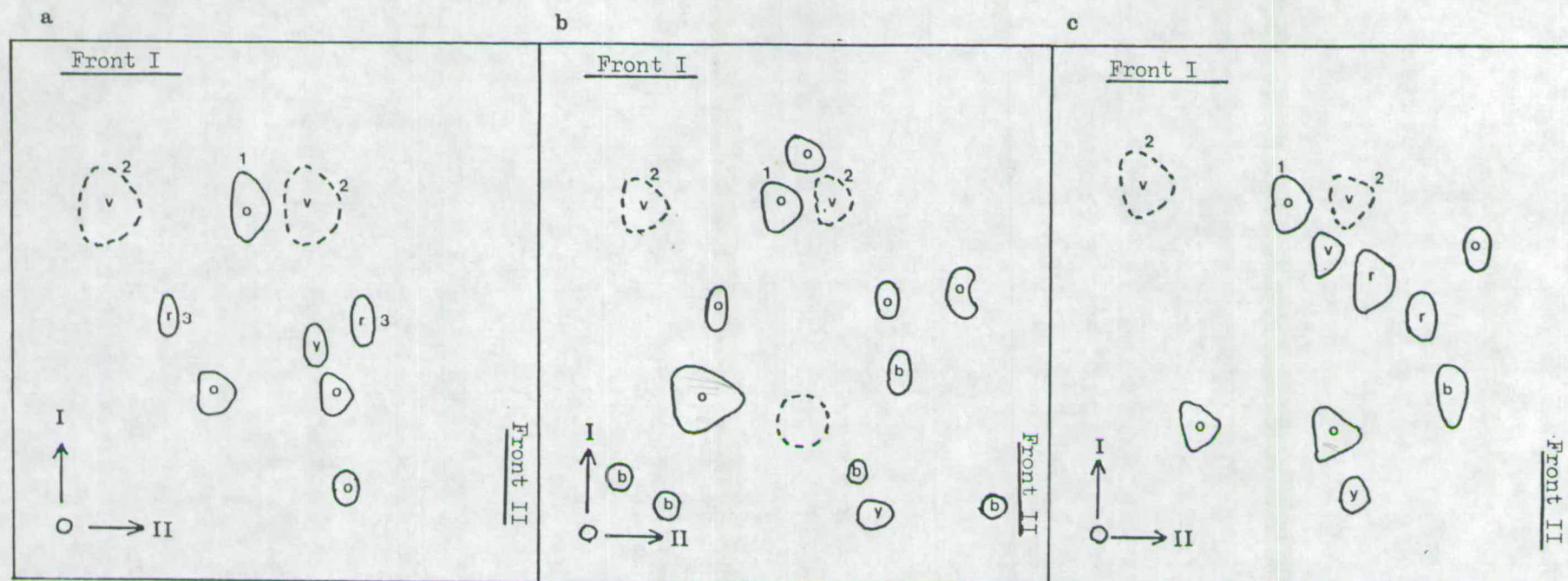


Fig. 3.1.1. TLC chromatograms of free phenolics released from their conjugated forms in an aqueous fraction of callus extract following [a] alkaline hydrolysis, [b] acid hydrolysis and [c] enzyme hydrolysis. TLC was performed on cellulose coated eluted with C_6H_6 -HOAc- H_2O (60:22:1.2) and 2% HOAc first and second dimensions respectively. Phenolics were visualized with a diazo spray reagent and 10% sodium carbonate. 1. Vanillic acid, 2. *cis* and *trans* ferulic acid, 3. *cis* and *trans* p-coumaric acid. Colour: b. brown, o. orange, r. red, v. violet and y. yellow. Dashed spot shows that the compound fluoresces under long UV light.

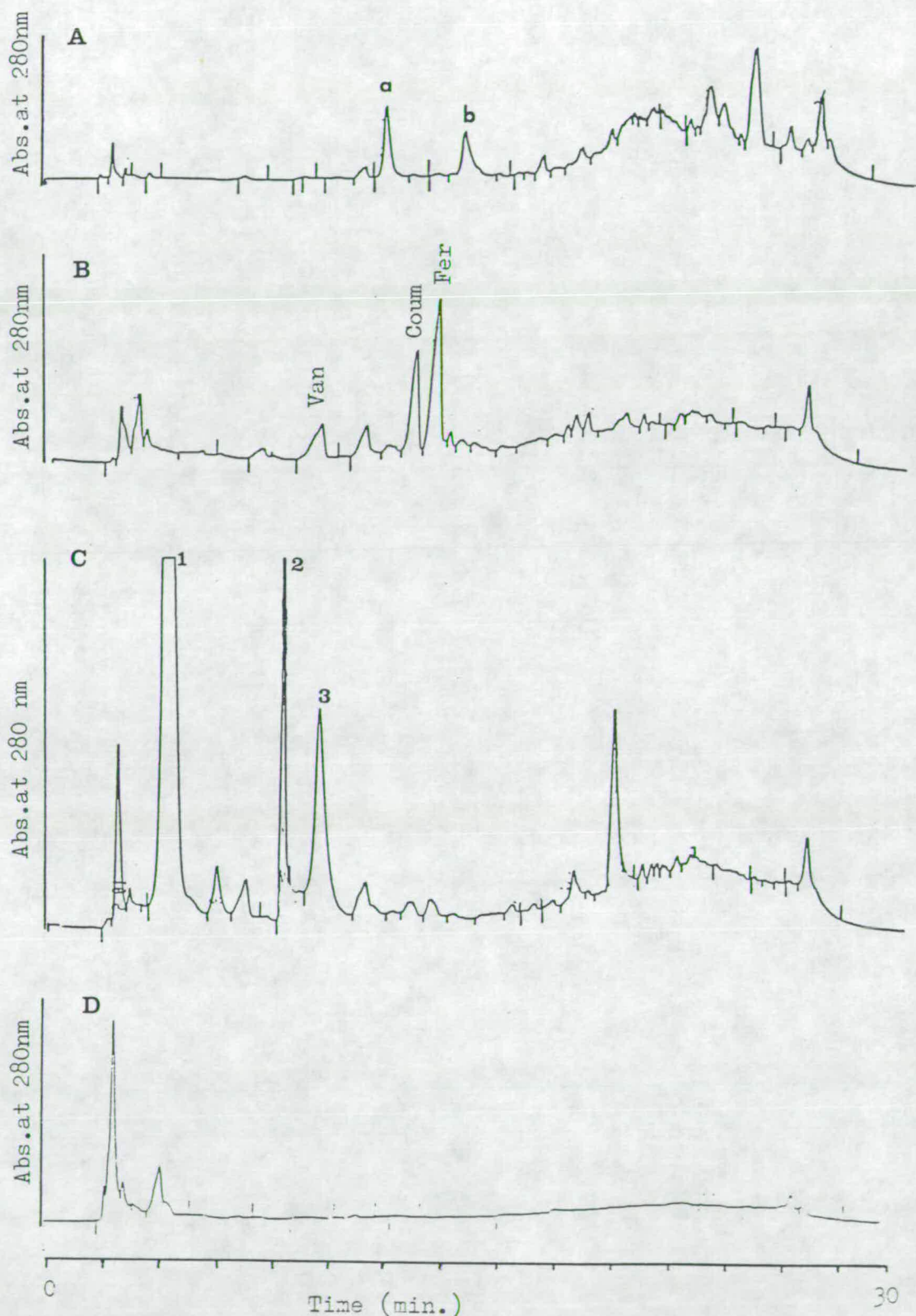


Fig. 3.1.2. HPLC chromatograms of (A) free phenolic fraction, (B) alkaline hydrolysis product, (C) acid hydrolysis product and (D) saponifiable cell wall phenolics. Peak a and b were residual conjugated phenolics extracted into EtOAc-Et₂O phase during liquid-liquid extraction. Van. vanillic acid, Coum: p-coumaric acid and Fer: ferulic acid. Peaks 1, 2 and 3 were the degradation products of sucrose during acid hydrolysis. HPLC method was as described in 2.3.4.

shows that there were no detectable saponifiable cell wall phenolics in callus tissue.

3.1.1.2. Physical appearance and growth of callus

Fig. 3.1.3 shows the physical appearance of callus grown on SH and MS media with the same levels of growth regulators. SH callus (Fig. 3.1.3.a) is friable while MS callus (Fig. 3.1.3.b) is compact. The major difference in composition between the media is the ammonium level. In SH medium, the ammonium level is 2.6 mM, while in MS medium it is 42.5 mM. A high concentration of ammonium has been shown to reduce the formation of organic anions which have an important role in the translocations of cations (Salsac *et al.*, 1987; Van Beusichem *et al.*, 1988).

The growth curves of the cultures are also different (Fig. 3.1.4). No marked lag phase was observed with SH callus. The FW and DW of SH callus increased gradually from d. 0 to d. 6 followed by a linear increase over 12 days with an average increase of 0.175g(FW) or 13.5mg(DW) *per day*. This culture reached the stationary phase at d.18. Between d.3 and d.12 the increase in cell number was $0.45 \cdot 10^6$ cells *per day* then after d.12 the cell division was 5X faster and reached the stationary phase at d.15. Although cell division ceased by d.15, the cells were still actively producing cellular materials, shown by the increase in FW and DW after d.15. In contrast MS callus grew slowly throughout the culture period. Despite the fact that the MS callus was still growing when SH callus had reached the stationary phase, its final FW, DW and cell number at d.30 were still lower than SH callus.

3.1.1.3. Accumulation of esters or O-glycosylated phenolics

Free phenolics and saponifiable cell wall phenolics were undetected (see 3.1.1.1.i and 3.1.1.1.v) and consequently the accumulation of these phenolics was not followed. Capsaicin which in the extraction method used will be in the free phenolic fraction was also not detected in these cultures under the conditions described. The accumulation of water soluble conjugated phenolics was followed by measuring the levels of phenolics in unhydrolysed extracts using HPLC. Although hydrolysis of an extract is a necessary prerequisite to the identification of the phenolic moieties of

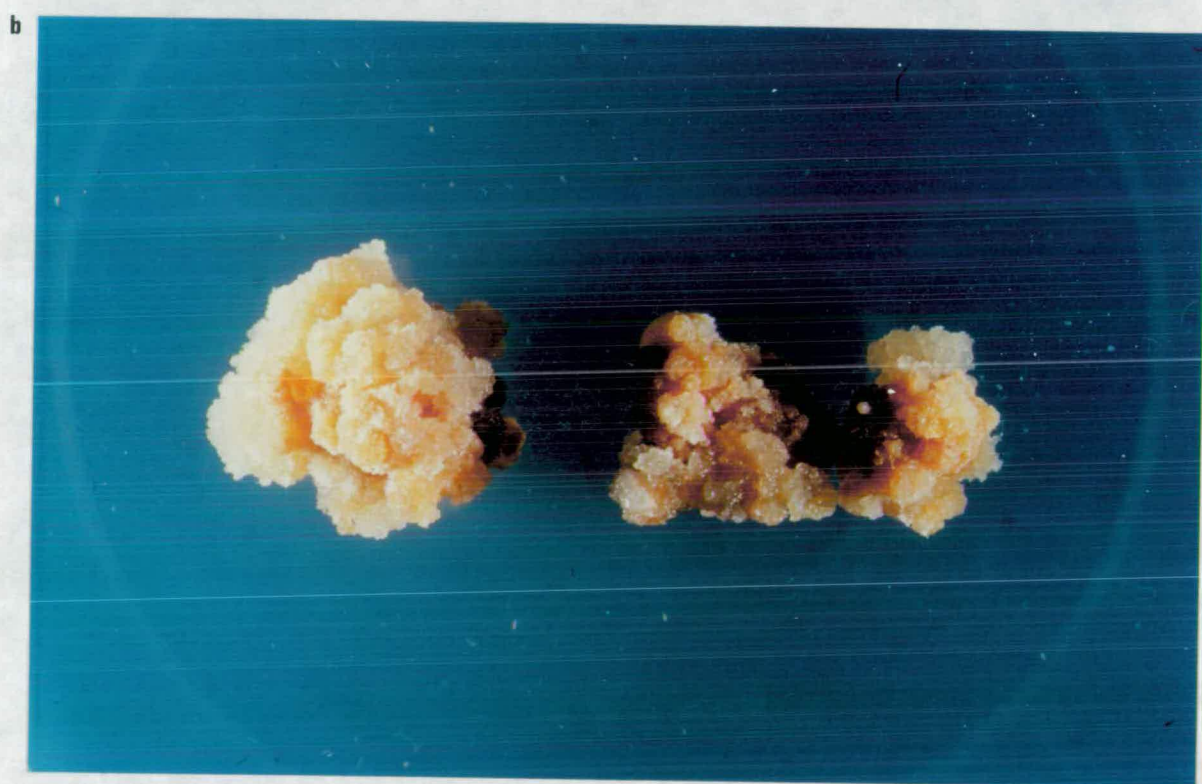
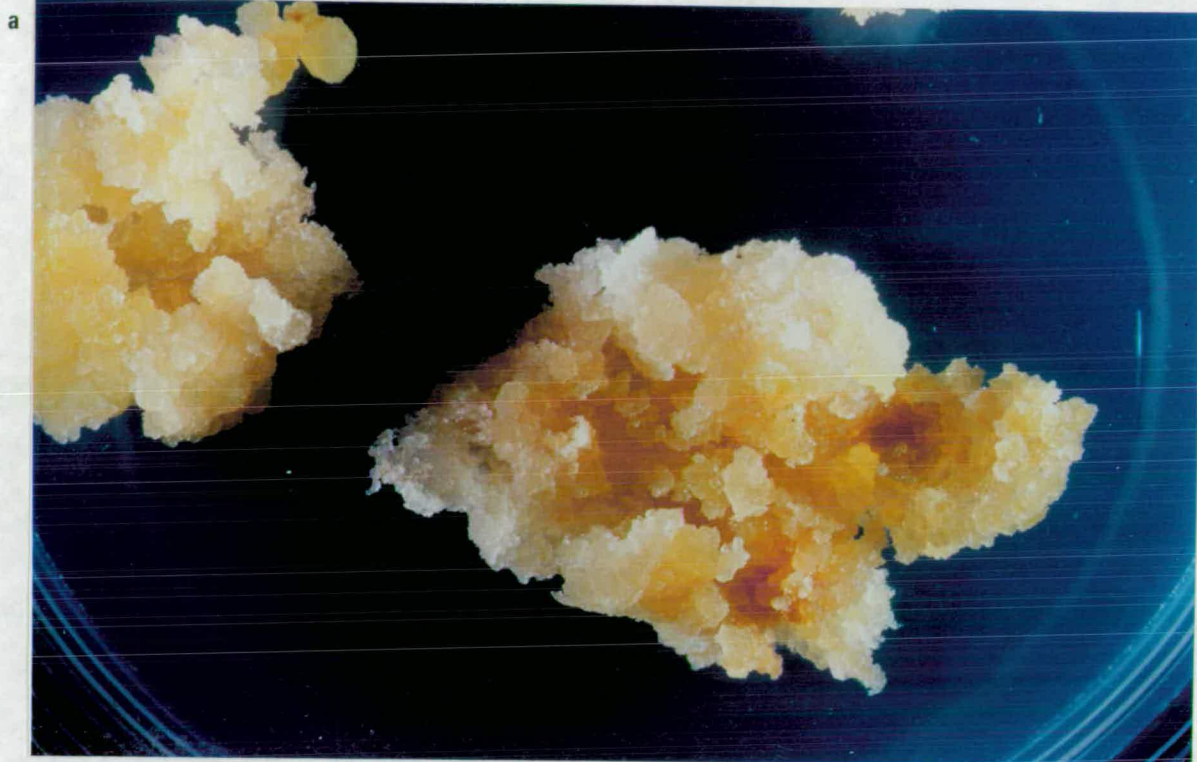


Fig. 3.1.3. Physical appearance of callus grown on SH (a) and on MS medium (b)

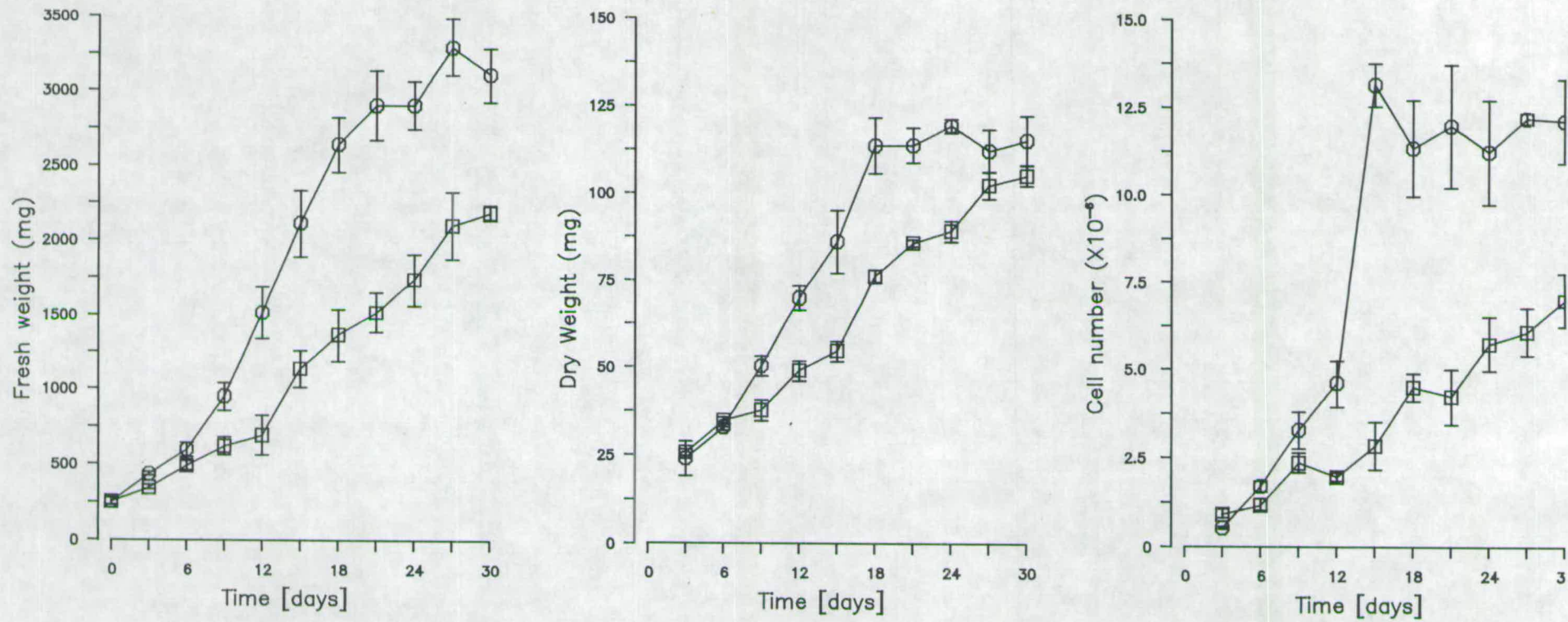


Fig. 3.1.4. Growth curves of SH callus [O] and MS callus [◇]. Bars represent SE of the mean of three replicates.

conjugated phenolics, it does make the measurement of the levels of endogenous phenolics difficult because some phenolics, especially ortho-dihydroxyphenolics are unstable in alkali. During acid hydrolysis, non-phenolic compounds are degraded and the degradation products interfere with the analysis of the compounds being studied. Decarboxylation of phenylpropanoid compounds also takes place during acid hydrolysis (Harborne, 1984). Evidence for the instability of phenolics during alkaline or acidic hydrolysis was also revealed in this experiment by the presence of the additional phenolics detected following enzymic hydrolysis. Emulsin (β -glucosidase from almond) on the other hand will mainly hydrolyse phenolics bound with glucose via a β -glucosidic bond. Phenolics bound to the other sugars may also be hydrolysed by the impurities in the enzyme preparation but less efficiently.

To measure the levels of conjugated phenolics, three pieces of callus [at least 250mg(FW) each] were extracted as described in 2.3.2.1. Samples were prepared by dissolving the residues of the aqueous fractions in 80% HPLC grade MeOH (3g(FW)tissue.ml⁻¹). Phenolics were separated and quantified by injecting 10 μ l of sample to an HPLC chromatograph (see 2.3.4). Here, there was a problem in dissolving these compounds due to the presence of sticky materials in the extract. Certain phenolics identified following the alkaline or acid hydrolysis (see 3.1.1) might not be detected in their conjugated form, as the levels of the compounds in the samples could be below the limit of detection. The other possibility was that two or more phenolic moieties bound to a single sugar form a compound with a UV spectrum different from the individual phenolics.

At least 7 peaks were detected in extracts of both SH and MS callus (Fig. 3.1.5). The position of the hydroxy group in a phenolic will greatly affect the shape of the absorption spectrum and its maximum wavelength. The substitution of hydrogen on a hydroxyl or a carboxyl group with non-chromophore moieties will not significantly change the shape of the spectrum but it may shift the maximum wavelength slightly (see appendix). With the assumption that one phenolic was bound to one non-phenolic compound (usually a sugar) and that the single peak represents a single compound, tentative identifications were made.

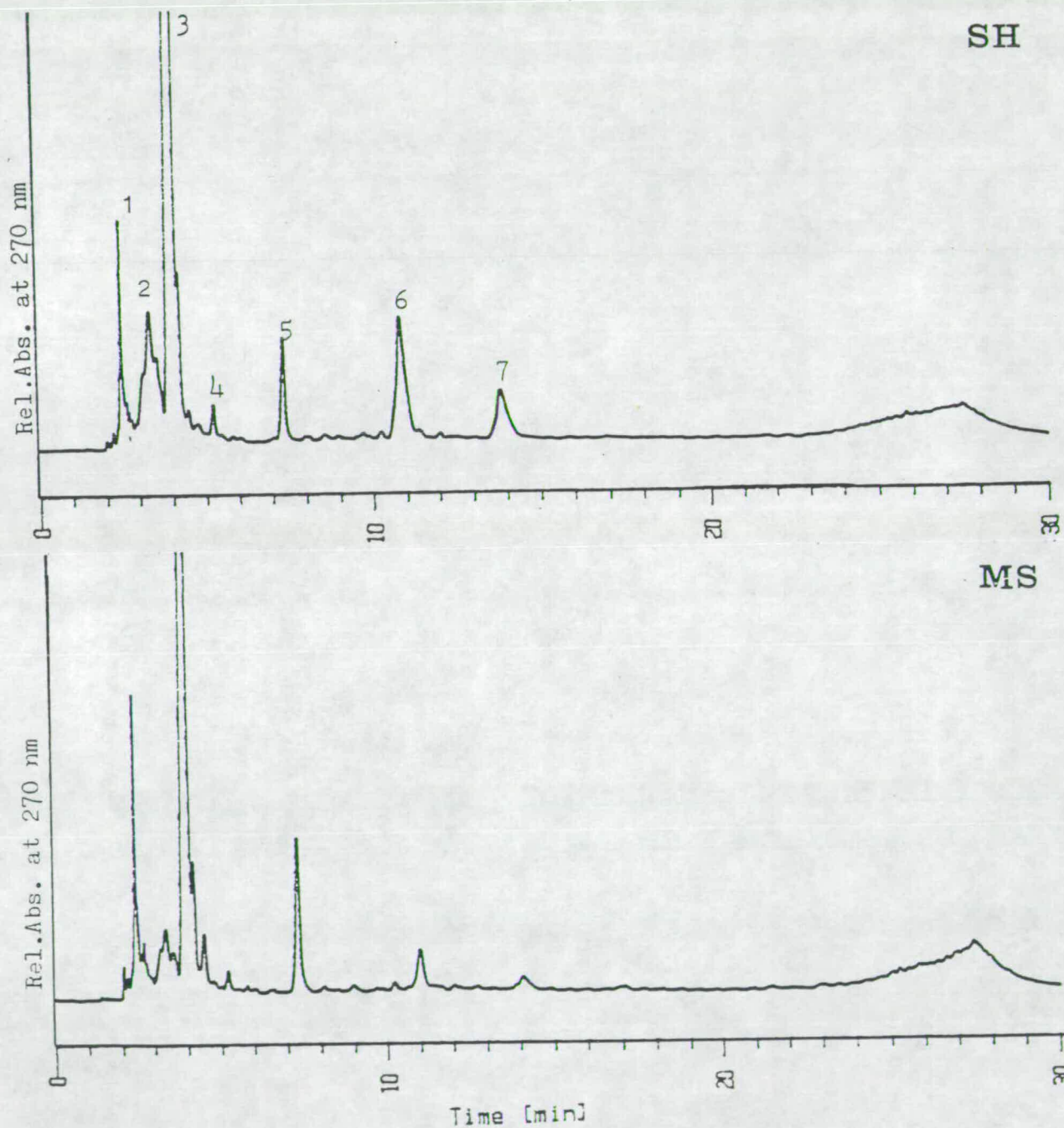
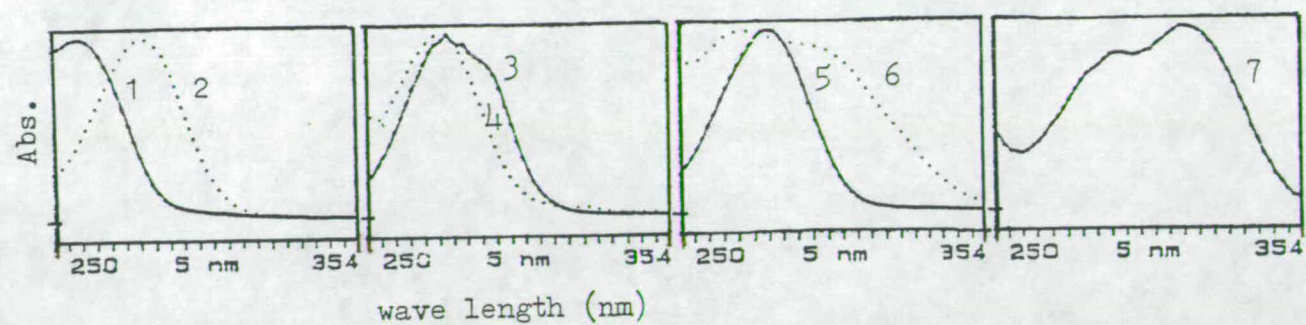


Fig. 3.1.5. HPLC chromatograms of the aqueous fraction of MS and SH callus extracts and the corresponding UV spectra of each peak. The HPLC method was as described in 2.3.4

The compound represented by peak-1 (phenolic-1) (Fig. 3.1.5) has a UV spectrum resembling that of p-hydroxybenzoic acid, this compound is possibly p-oxybenzoic acid-glycoside. Peaks 2, 4 and 5 all have the same type of UV spectrum which is typical of 3,4,5-trioxybenzoic acid. One of these compounds most commonly found in plants is gallic acid as a component of polyphenol (gallotannin) (Haslam, 1989). These three compounds are possibly 3,4,5-trioxybenzoic acid-glycosides. Phenolic-3 possibly has more than one phenolic moiety or the peak might be due to more than one compound with the same *R_t* values. Its maximum wavelength is close to phenolics-2, 4 and 5 but the shape of its UV spectrum is slightly different. The UV spectrum of peak-7 is typical of 3,4-dioxycinnamic acid and this compound is possibly 3,4-dioxycinnamic acid-glycoside. When vanillin was heated in alkaline solution at 104°C for 6h., it produced a small amount of compound with a UV spectrum similar to the UV spectrum of phenolic-6 [Bladon, unpublished observation]. Phenolic-6 is possibly a glycoside of a vanillin related phenolic. Since the same substitution pattern of phenolic will also give the same colour with the diazo-reagent, the aglycone of this glycoside is possibly the large orange spot on the TLC which is the acid hydrolysis product of the glycoside (Fig. 3.1.1.b).

Fig. 3.1.6 shows the accumulation of conjugated phenolics in MS and SH cultures over the culture period. The compounds of peaks-2, 4 and 5 (phenolics-2, 4 and 5 as described in Fig. 3.1.5) in SH cultures were accumulated actively in the early linear phase (d.6 to d.12), the levels decreased during the late linear phase (d.12 to d.15) and remained at a low level in the stationary phase. In MS callus, a similar pattern of accumulation was observed, with the maximal levels at d.9 and phenolics-2 and 4 were reaccumulated at the end of the culture period.

Phenolic-3 is the most abundant phenolic in both cultures. This phenolic was accumulated actively in SH callus during the linear phase (d.6 to d.15), then the level decreased after the culture reached the stationary phase (d.15 to d.30). In MS cultures, the level of this phenolic was higher at d.9 and d.24 than at the other periods of cultures, but its amount *per callus* always increased throughout the culture period. The level of phenolic-1 in SH cultures was high in the period of slow growth (d.3 to

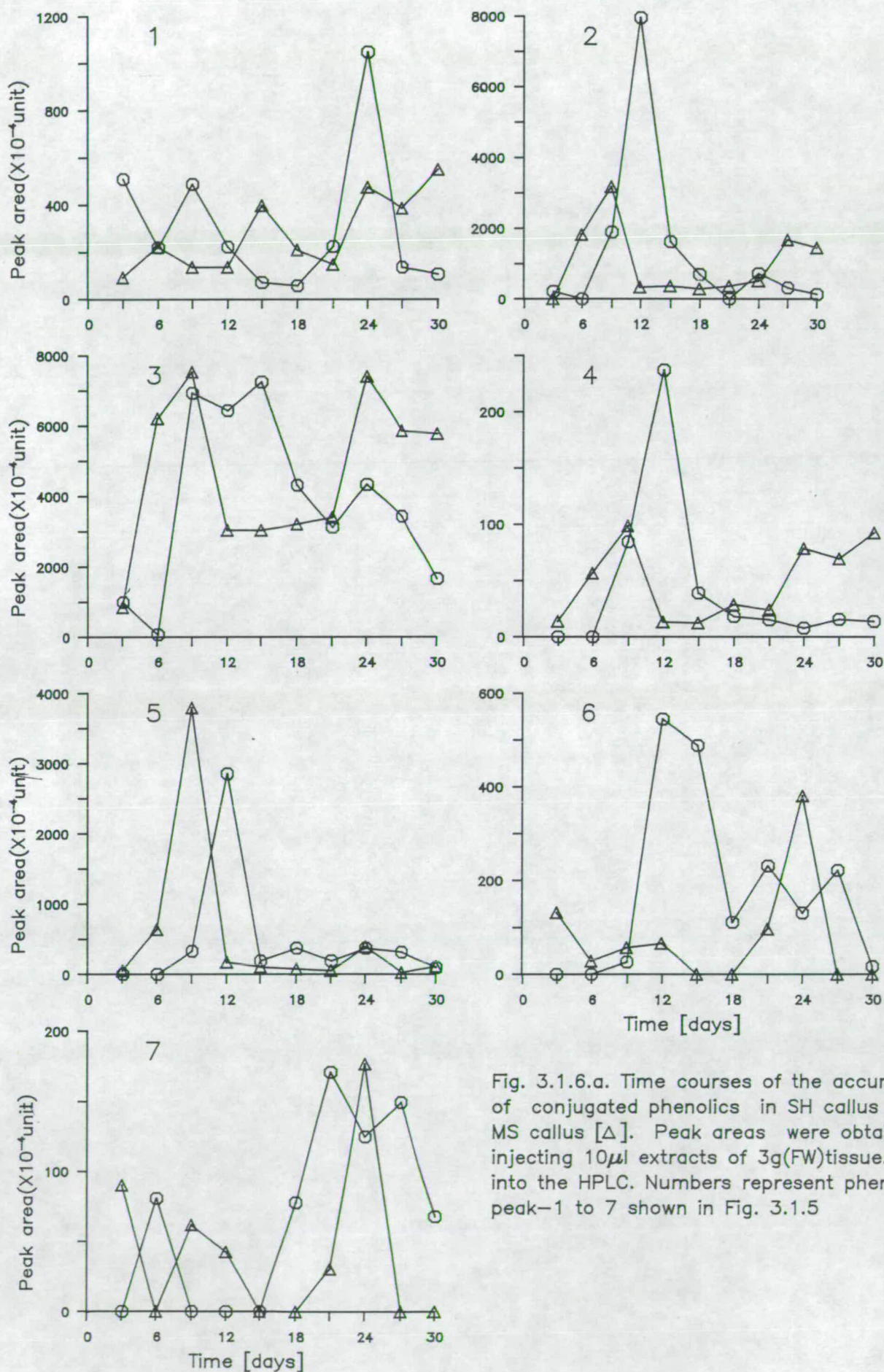


Fig. 3.1.6.a. Time courses of the accumulation of conjugated phenolics in SH callus [O] and MS callus [Δ]. Peak areas were obtained by injecting 10 μ l extracts of 3g(FW)tissue.ml⁻¹ into the HPLC. Numbers represent phenolics of peak-1 to 7 shown in Fig. 3.1.5

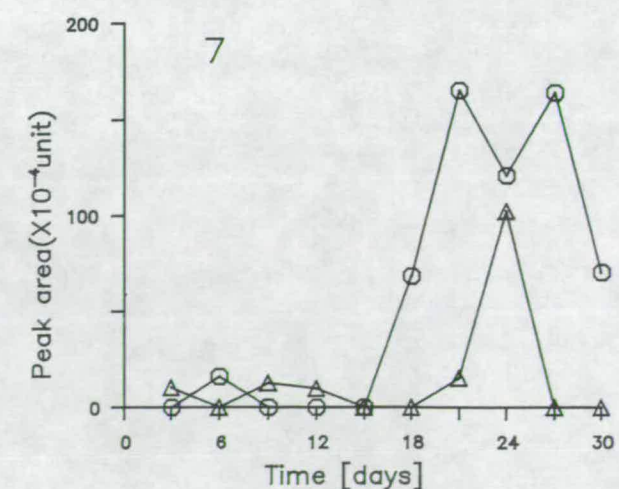
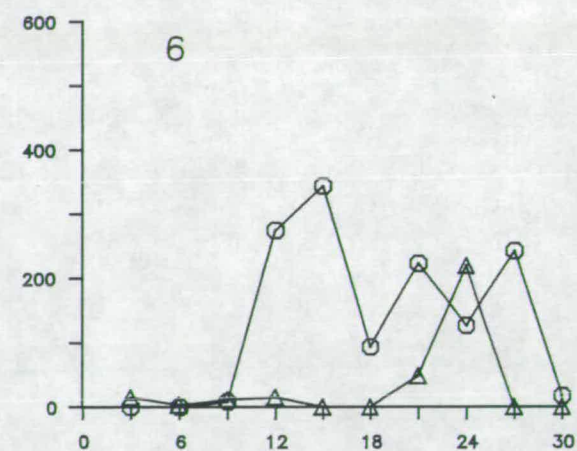
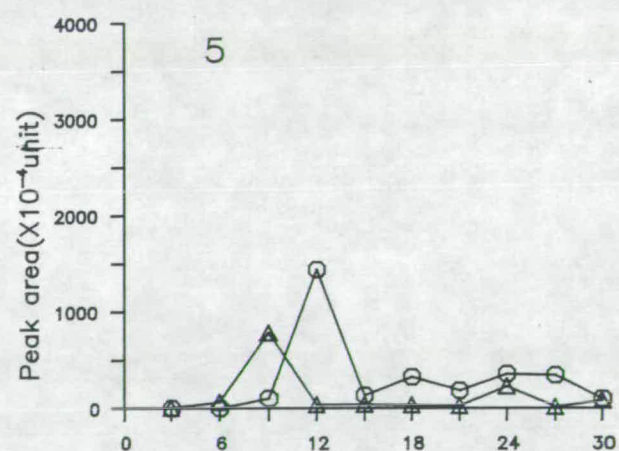
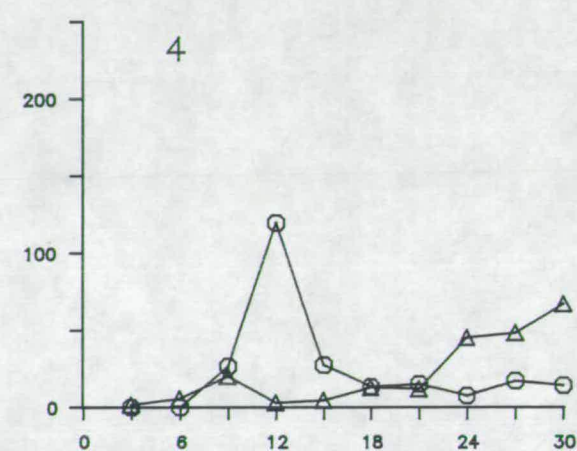
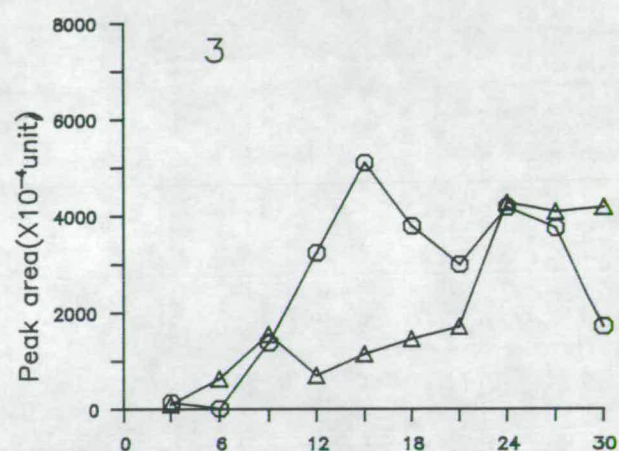
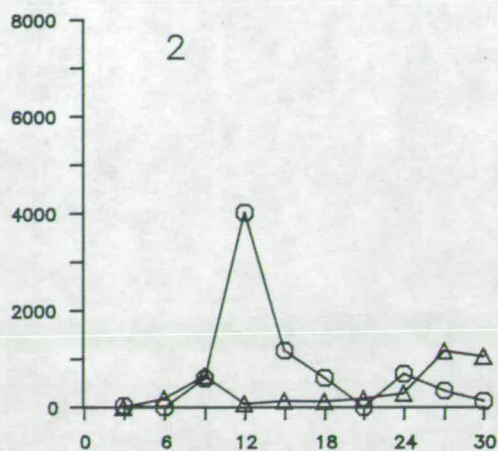
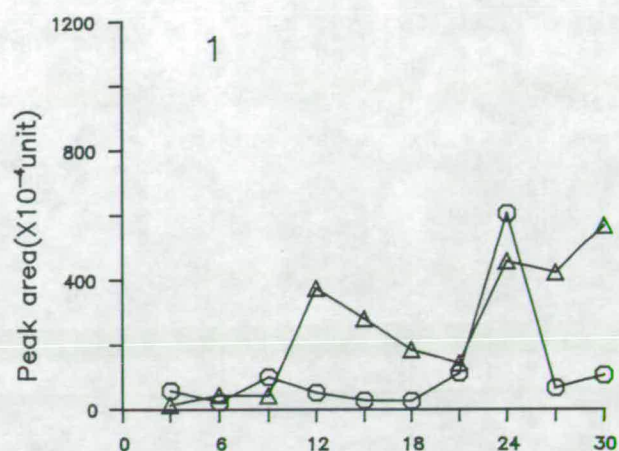


Fig. 3.1.6.b. Time courses of the accumulation of conjugated phenolics in SH callus [O] and MS callus [Δ]. Peak areas were obtained by injecting 10 μ l extracts of 1 callus.ml⁻¹ into the HPLC. Numbers represent phenolics of peak-1 to 7 shown in Fig. 3.1.5

d.9) and then decreased during the linear phase. This phenolic was reaccumulated at the early stationary phase (d.18 to d.24) then the level decreased again by the end of the culture period. In slow growing MS cultures, the level of this phenolic increased slowly throughout the culture period.

In SH cultures, phenolic-6 was accumulated to a high level in the rapid growth period (d.12 to d.15) then the level decreased after cell division ceased. The level of this phenolic in MS cultures was low from d.3 to d.18 then increased rapidly between d.18 to d.24 and decreased by the end of the culture period (d.24 to d.30). In MS cultures the same pattern of accumulation was observed with phenolic-7 (3,4-dioxycinnamic acid-glycoside). In SH callus, this compound was at a low level in the linear phase (d.9 to d.15) and at a higher level during the period of slow growth (d.6 and d.18 to d.24). As with the other phenolics, the level of this compound also decreased by the end of the culture period.

Fluctuations in the amounts of these phenolics per callus were also observed (see Fig. 3.1.6.b). This is possibly due to an interconversion between one phenolic with another. In SH cultures, all of these conjugated phenolics disappeared by the end of the culture period. These phenolics were possibly incorporated into MeOH insoluble materials in the cell wall. Although MS callus grew much more slowly than SH callus, it only accumulated a higher level of phenolic-5.

3.1.1.4. Accumulation of lignin

The level of lignin in callus tissue was measured according to the method described in 2.3.5.2. Fig. 3.1.7. shows the accumulation of lignin in both MS and SH cultures. In SH cultures, the lignin level did not increase in the active growth period (d.3 to d.12) (see Fig. 3.1.7.A). However, there was a small increase in the lignin level when cell division had ceased (d.15). This level remained constant until d.24, then increased again at the end of the culture period (d.24 to d.27). In MS cultures, the lignin level was high at d.3 then decreased until d.9 followed by a small fluctuation until the end of the culture period.

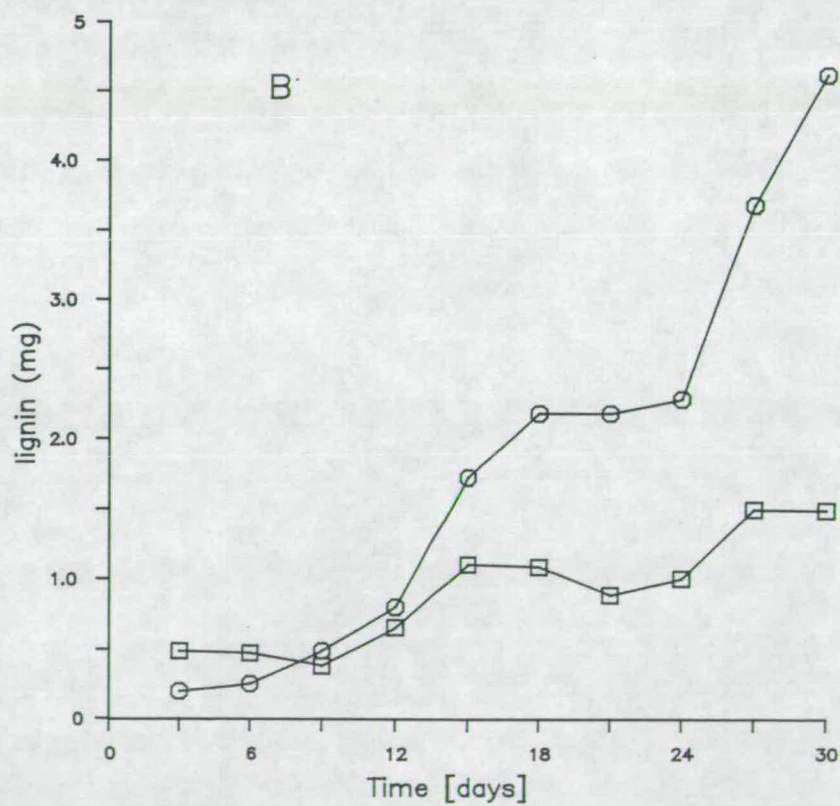
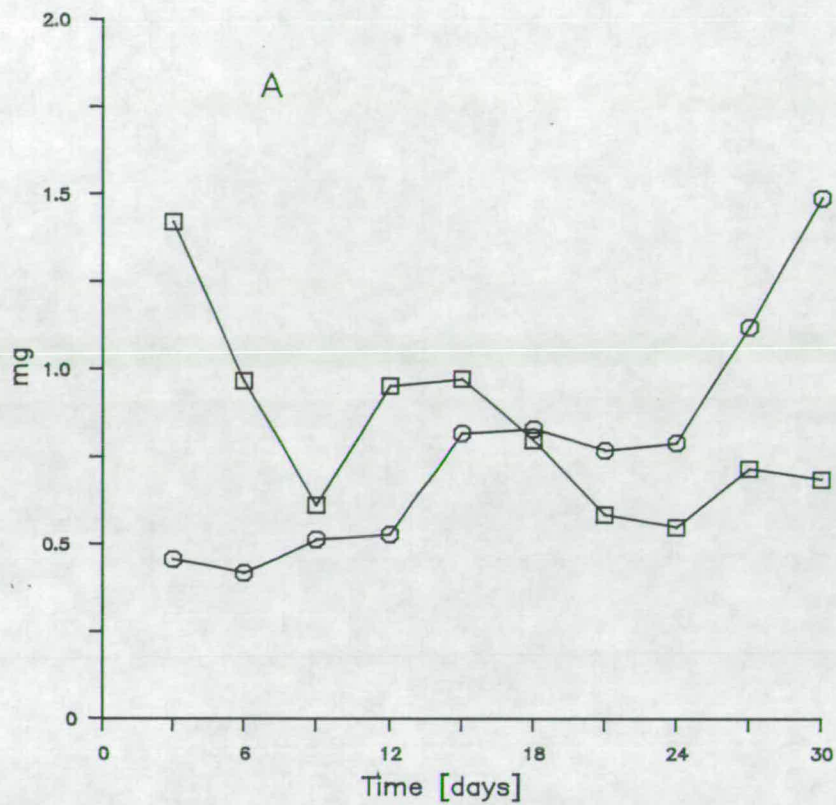


Fig.3.1.7. The accumulation of lignin in callus cultures of *Capsicum frutescens* grown in SH (O) and MS (□) medium respectively. A. expressed as *per g*(FW) and B. as *per callus*.

The lignin level in MS cultures was higher than in SH cultures between d.3 and d.15, in the later stages it was higher in SH cultures. The amount of lignin *per* callus in both cultures either increased or remained constant (Fig. 3.1.7.B). In callus cultures, lignin seems to be the end product of metabolism and forms a major sink for phenolic intermediates of capsaicin biosynthesis. Most conjugated phenolics which disappeared at the end of the culture period were possibly incorporated into lignin.

3.1.2. In Suspension Cultures

The capsaicin produced in pepper cultures is released into the medium (Yeoman *et al.*, 1980; Lindsey and Yeoman, 1983). Therefore it is understandable that capsaicin was not detected in callus tissue in this experiment (3.1.1.1.iii). On the otherhand, it is difficult to extract phenolics from the solid medium (agar) efficiently. However, by using cell suspension cultures, the phenolics and capsaicin in the liquid medium can easily be extracted.

3.1.2.1. Physical appearance and growth of suspension cultures

Fig. 3.1.8 shows the physical appearance of pepper cell suspension cultures grown in MS and SH media. As with callus, SH cultures were healthier in appearance than MS cultures. Although the MS cultures were not contaminated, after two weeks from subculture, an ammoniacal odour was noted.

Growth of cultures was followed by measuring the FW of cells produced. Fig. 3.1.9 shows the growth curves of cell suspension cultures grown in MS and SH media. In MS cultures, a lag phase was observed between d.0 and d.9. From d.9 to the end of the culture period, the cultures grew very slowly with an average increase in FW of 0.14g.d^{-1} . There was no lag phase observed in the SH cultures. Between d.0 and d.9 the cultures grew slowly with an average increase in FW 0.08g.d^{-1} . From d.9 to the end of the culture period, cultures grew rapidly with an average increase in FW 0.7g.d^{-1} . By the end of the growth period, cultures in both media had not reached a



Fig. 3.1.8. Suspension cultures of *Capsicum frutescens* grown in MS and SH media. Cultures were 15d. from subculture.

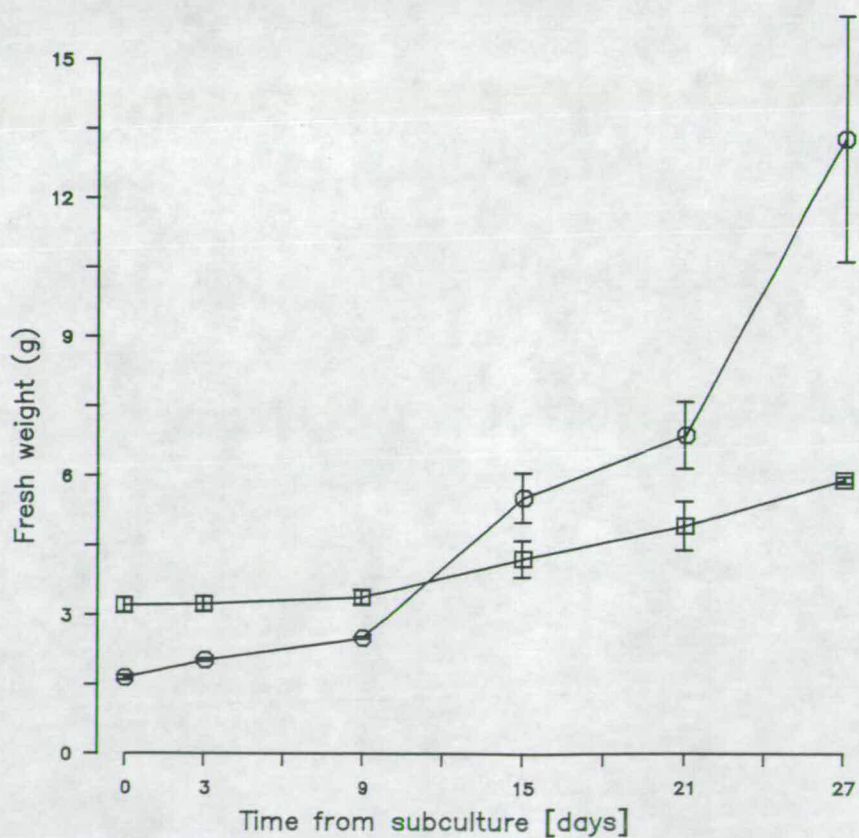


Fig. 3.1.9. Growth curve of suspension cultures of *Capsicum frutescens* grown in SH [O] and MS [□] media. Bars represent SE of the mean of three replicates

stationary phase.

3.1.2.2. Phenolics in the medium

After removal of cells from the cultures by filtration, the phenolics in the medium were extracted according to the method described in 2.3.2.2. The phenolic extracts obtained were taken up in 1.0ml MeOH and 20 μ l samples were injected into an HPLC chromatograph. Fig.3.1.10 shows the HPLC chromatograms and the UV spectra of the corresponding peaks of the phenolics present in the medium of MS and SH cultures. Both cultures accumulated a similar pattern of phenolics in the medium. No capsaicin was detected in either culture. Although there was a peak in the chromatogram of the phenolic extract of MS medium with an Rt value close to capsaicin (Rt 23 in Fig. 3.1.10), but this compound displayed a different UV spectrum from capsaicin (data not shown).

The UV spectrum of phenolic-1 (Fig.3.1.10) is typical of 3,4-dioxybenzaldehyde (see appendix) with an Rt value close to protocatechuic aldehyde. The other phenolics have a similar type of UV spectra, which is similar to the UV spectrum of m-coumaric acid (see appendix), however this compound is rarely found in higher plants. Only further detailed analysis with NMR and mass spectrometry would reveal the chemical nature of these compounds.

Fig. 3.1.11 shows the pattern of accumulation of phenolics in both media over the time course. The amount of phenolic-1 in SH cultures increased from d.0 to d.21 then decreased rapidly until the end of the culture period. In MS cultures, it increased from d.0 to d.15 then decreased and remained constant after d.21. Phenolics-2, 3 and 4 in SH cultures showed the same pattern of accumulation, their levels increased from d.0 to d.9 or 15 then remained constant until the end of the culture period. In MS cultures, the accumulation pattern of phenolic-4 was the same as in SH cultures, while phenolics-2 and 3 were present at low levels between d.0 and d.9 or 15 then increased until the end of the culture period. Phenolics-5 and 6 were the most prominent phenolics in the medium of both cultures. These phenolics were present at low levels during the period of slow growth (d.0 to d.9). In SH cultures, the levels

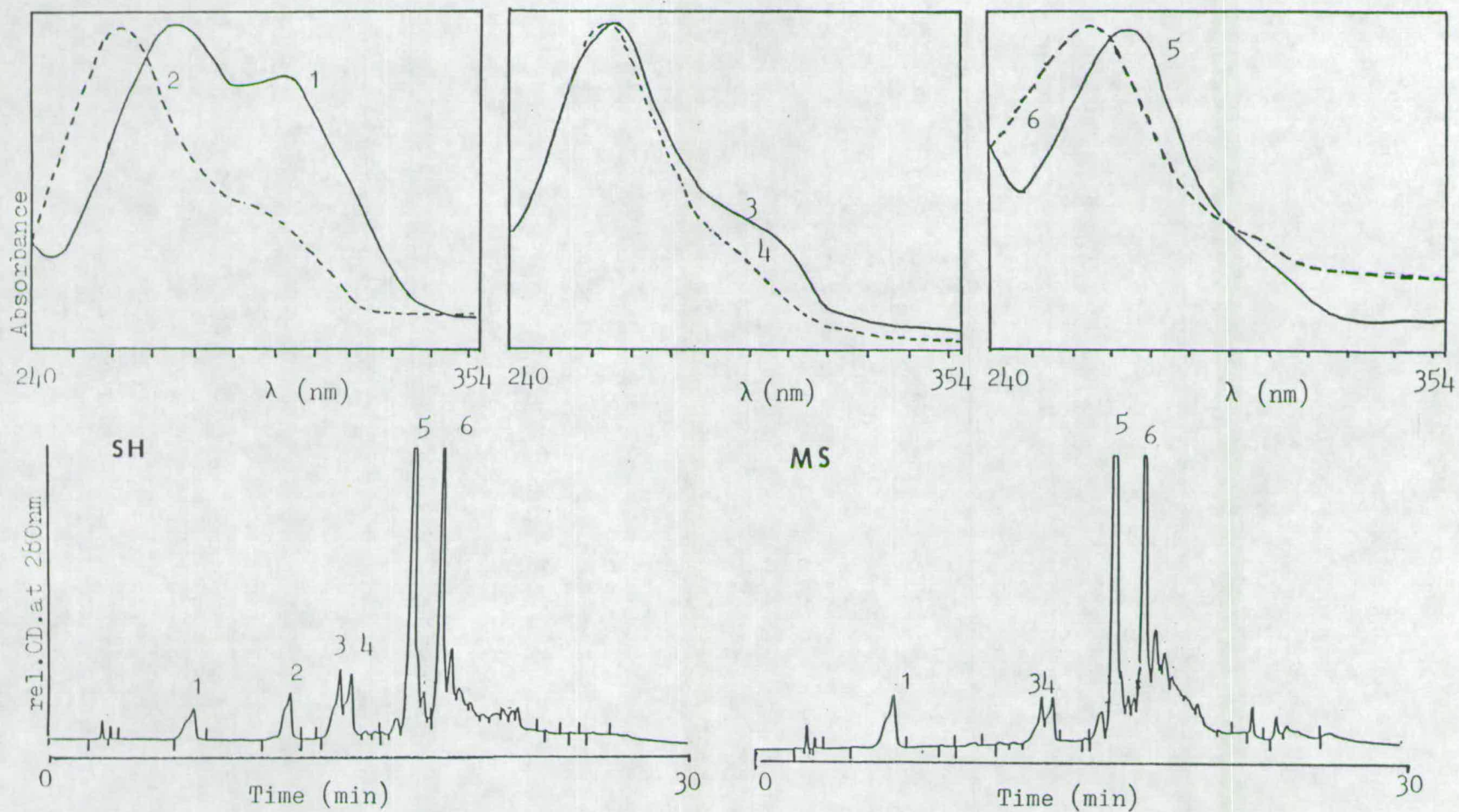


Fig. 3.1.10. HPLC chromatograms of free phenolics released in to the medium of MS and SH cultures and the UV spectra of the corresponding peaks. The HPLC system was as described in 2.3.4.

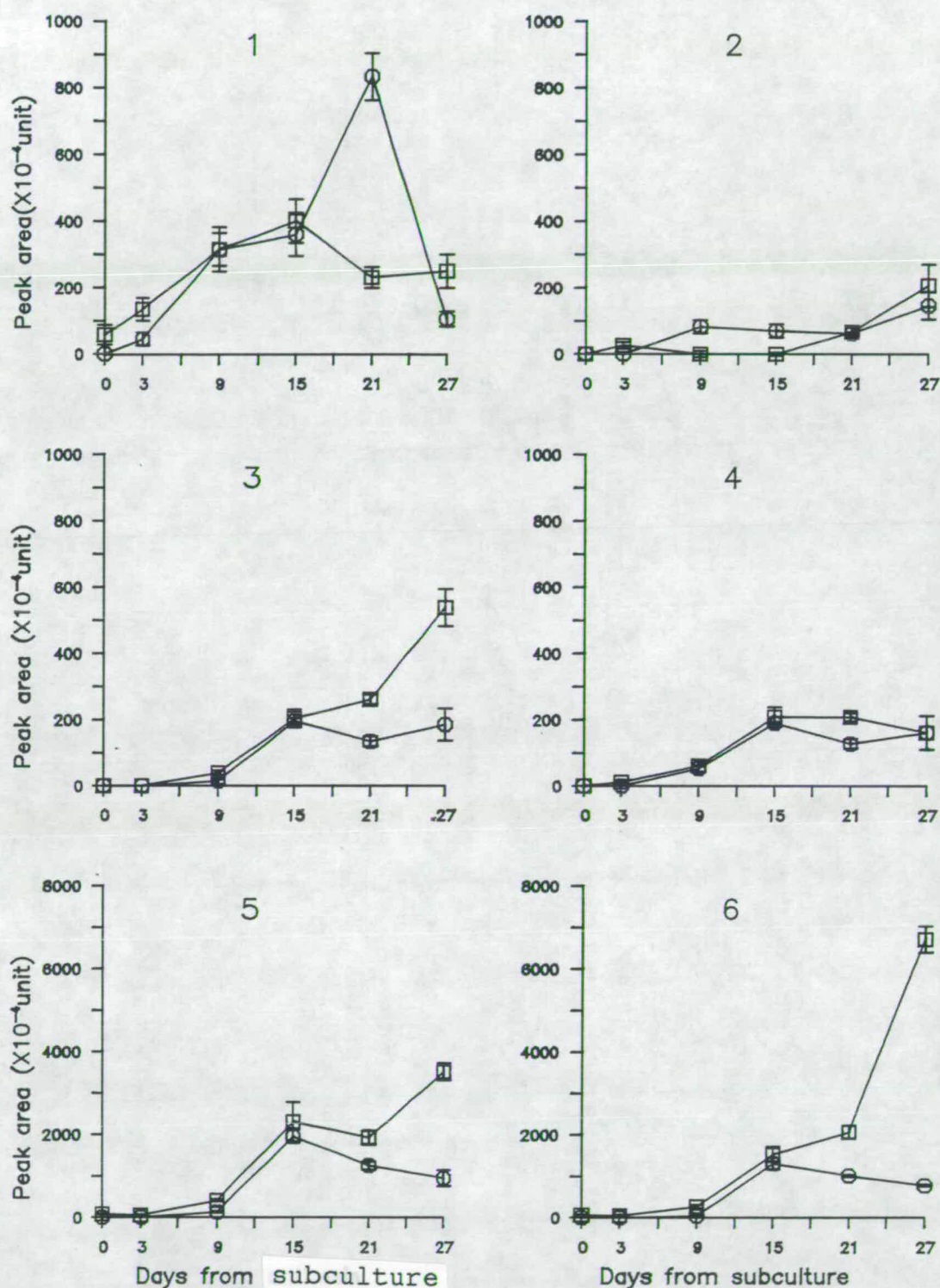


Fig. 3.1.11. The accumulation of free phenolics in the medium of MS [□] and SH cultures [○]. Peak areas were obtained by injecting 20 μ l extract of 1.0ml per culture into the HPLC chromatograph. HPLC method was as described in 2.3.4. Bars represent SE of the mean of three replicates. Numbers represent the corresponding compounds shown in Fig. 3.1.10

increased in the early linear phase (d.9 to d.15) then decreased until the end of the culture period. The levels of these phenolics in MS cultures always increased after d.9.

Only the concentration of phenolic-1 was higher in SH cultures than in MS cultures (at d.21). Although after d.12 the MS cultures had less biomass than the SH cultures, the levels of the other phenolics were always higher in MS cultures. Phenolics-1, 5 and 6 in SH cultures decreased by the end of the culture period, these compounds were possibly taken up and turned over by the cells.

3.1.2.3. Phenolics in the cells

As in callus cultures, there were no detectable free phenolics in the cells. However, significant amounts of esterified or glycosylated phenolics were detected. Phenolics in cells were extracted and fractionated according to the method described in 2.3.2.1. Samples of conjugated phenolics were dissolved in 1.0ml of 80% MeOH per 3.0g(FW) tissue and 15 μ l applied onto the HPLC. Fig. 3.1.12 shows the HPLC chromatograms and the UV spectra of the corresponding peaks of conjugated phenolics in MS and SH cultures. Phenolics-1, 3 and 4 have the same UV spectra as phenolic-1 in callus (see Fig. 3.1.5) and are typical of p-OH-benzoic acid-glycosides (see Appendix). Phenolic-2 is possibly the same as the major conjugated phenolic in callus (phenolic-3 in Fig. 3.1.5). Phenolics-7 and 8 possibly have the same phenolic moieties as phenolics-6 and 7 in callus (see Fig. 3.1.5). As the *Rt* values are higher than those found in callus, these phenolics are possibly bound to different types or lesser number of sugar monomers. Phenolics-5 and 6 are possibly the glycosides of the two most prominent phenolics in the medium (see Fig. 3.1.10).

The levels of all conjugated phenolics in both cultures increased in the slow growth period (d.0 to d.3) then either increased or remained constant between d.3 and d.21 and disappeared by the end of the culture period (d.21 to d.27) (see Fig. 3.1.13). There were no large differences in the levels of conjugated phenolics in either culture. By the end of the culture period, the carbon source in the medium was limiting growth. At this stage, the cultures may use the sugars bound to phenolics as a carbon

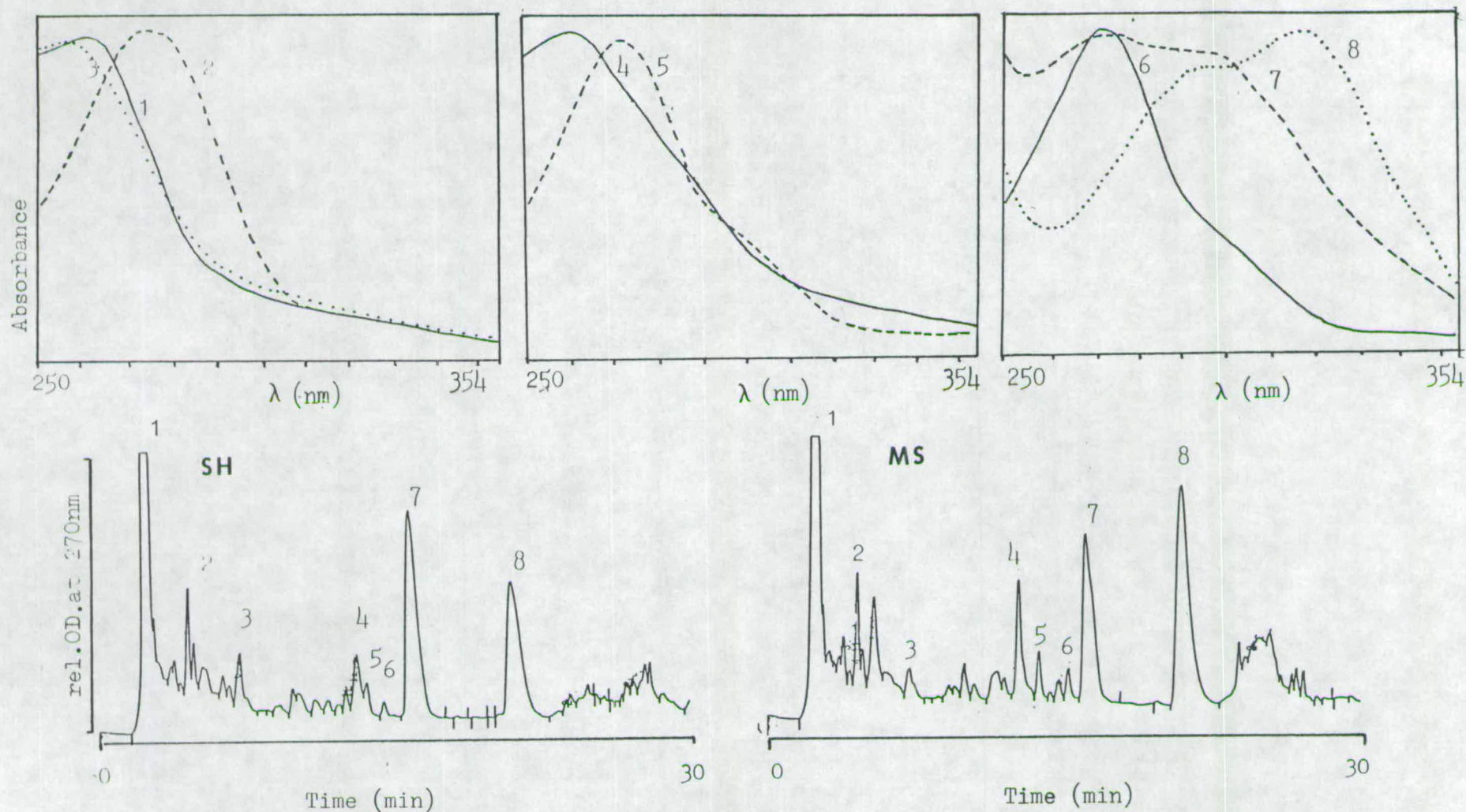


Fig. 3.1.12. HPLC chromatograms and UV spectra of the corresponding peaks of water soluble conjugated phenolics in suspended cells of *Capsicum frutescens* grown in MS and SH media. The HPLC method was as described in 2.3.4 with a slight modification to the solvent gradient and detection wavelength.

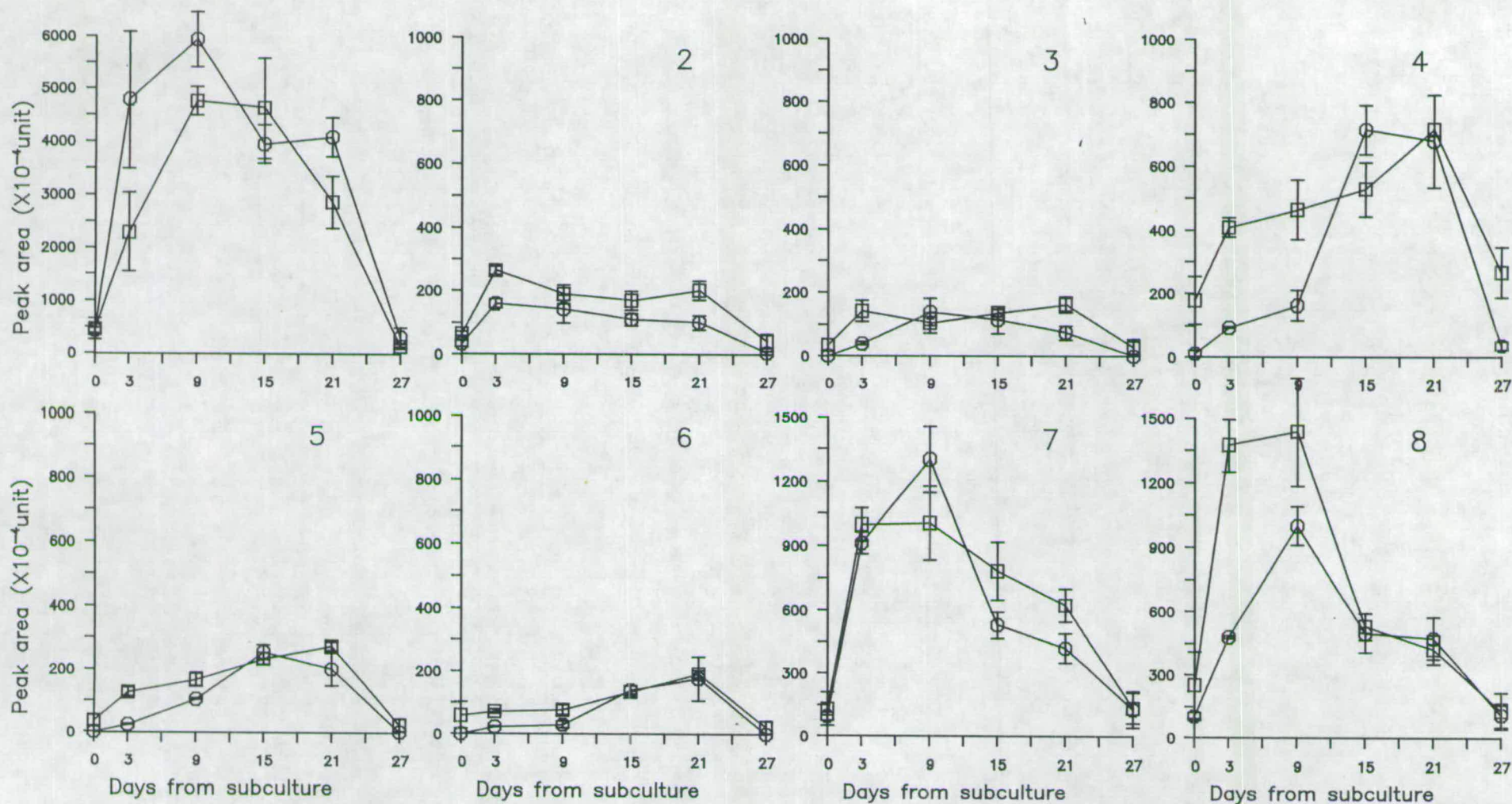


Fig. 3.1.13. The accumulation of conjugated water soluble phenolics in suspended cells of *Capsicum frutescens*. Peak areas were obtained by injecting 15 μ l extracts of 1.0ml *per* 3g(FW) of cells into the HPLC chromatograph. [O] for SH cultures and [□] for MS cultures. Numbers represent the corresponding compounds shown in Fig. 3.1.12. Bars represent SE of the mean of three replicates.

source and incorporate the phenolics released from their bound form into lignin.

3.1.2.4. The accumulation of lignin in suspension cultures

Fig. 3.1.14 shows the accumulation of lignin in MS and SH cultures. The amount of lignin *per g(FW)* in both cultures remained constant throughout the culture period. In MS cultures, it was approximately 3X higher than in SH cultures. The amount of lignin *per culture* in both cultures always increased throughout the culture period and there was always more lignin in MS cultures than in SH cultures.

Since capsaicin was not detected in these cultures, it is impossible to relate the accumulation of phenolics in both cells and medium with the synthesis of capsaicin. Lignin seems to be the major sink for phenolics in these pepper cultures, as it was present at mg levels and the amount *per culture* kept increasing.

3.1.3. The Effect of Sucrose Level on Growth and Accumulation of Phenolics in Cultures

It is generally believed that high sucrose concentrations increase the production of phenolics (Ibrahim, 1987). Indeed, it has been shown that the level of sucrose in the medium can influence the accumulation of a variety of secondary metabolites (Knobloch *et al.*, 1982; Fowler and Stepan-Sarkissian, 1985). As one of the objectives of this series of experiments was to study the relationship between phenolic metabolism and capsaicin synthesis, it was necessary to induce capsaicin accumulation in these cultures. In this experiment, the effect of sucrose concentrations on the accumulation of phenolics and capsaicin in pepper cultures was investigated.

3.1.3.1 . The effect of sucrose on growth

In this experiment, cultures were initiated using the same amount of inoculum ^[2g FW] in ^{50 ml of} SH medium containing 0, 1, 3, 5 and 7% sucrose. After 21d. when the control cultures (3% sucrose) were still growing and accumulating free phenolics in the medium (see 3.1.2.2 and Fig. 3.1.11), the cultures were harvested. and the FW and DW measured. Table 3.1 shows the effect of sucrose level on the final FW and DW

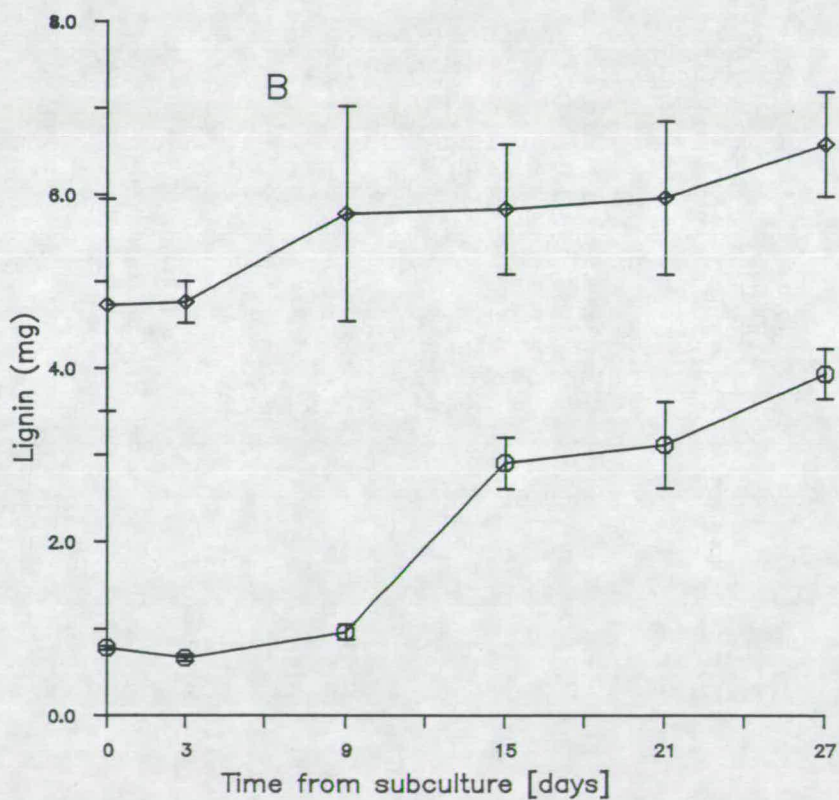
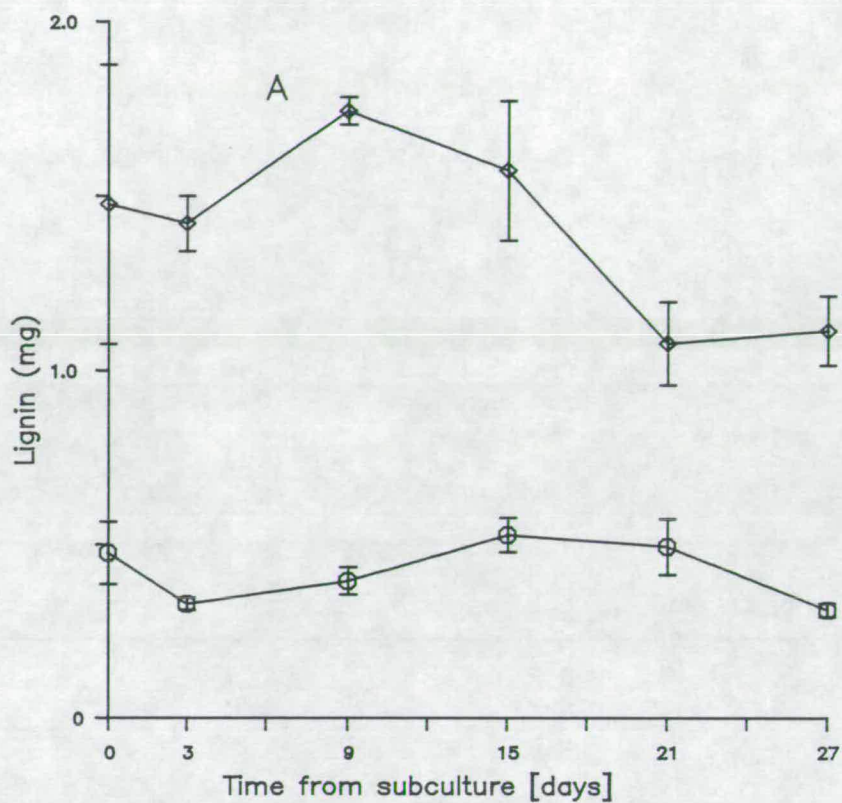


Fig.3.1.14. The accumulation of lignin in suspension cultures of *Capsicum frutescens*, in SH (O) and MS (◇) medium respectively. A, expressed as *per g*(FW) cell and B, *per culture*. Bars represent SE of the mean of three replicates.

of the cells. At 0% sucrose the cultures had grown slightly, possibly by using endogenous carbohydrates as a carbon source. At 1% to 5% the final FWs of cultures were similar, but their DWs were higher in cultures with higher sucrose levels. Although the final FW of cultures at 7% sucrose was lower than at 5%, the DW was similar.

Table 3.1. The effect of sucrose levels in the medium on FW and DW of cultures

% Sucrose	FW \pm SE (g)	DW \pm SE (mg)
0	6.01 \pm 0.49	163 \pm 11
1	12.25 \pm 1.02	436 \pm 23
3*	13.29 \pm 0.97	810 \pm 62
5	12.90 \pm 0.59	916 \pm 23
7	10.23 \pm 0.30	920 \pm 20

Note: * control

3.1.3.2. The effect of sucrose on phenolics released into the medium

The accumulation of phenolics in the medium was greatly affected by the sucrose level (Fig. 3.1.15). At 0% sucrose, only phenolic-1 (see Fig. 3.1.10) was produced. At 1% sucrose, the release of phenolics-3, 4 and 6 was the same as in control, and phenolic-5 was lower than the control. Phenolic-2 on the other hand was higher in cultures with 1% sucrose than in the control. At higher sucrose levels (5 and 7%) only phenolics-5 and 6 were detected in the medium at lower concentrations than the control. It is obvious that sucrose levels above 3% reduced the accumulation of free phenolics in the medium. Excess sugars in the cells were possibly used to glycosylate phenolics. No capsaicin was detected at any of the sucrose levels.

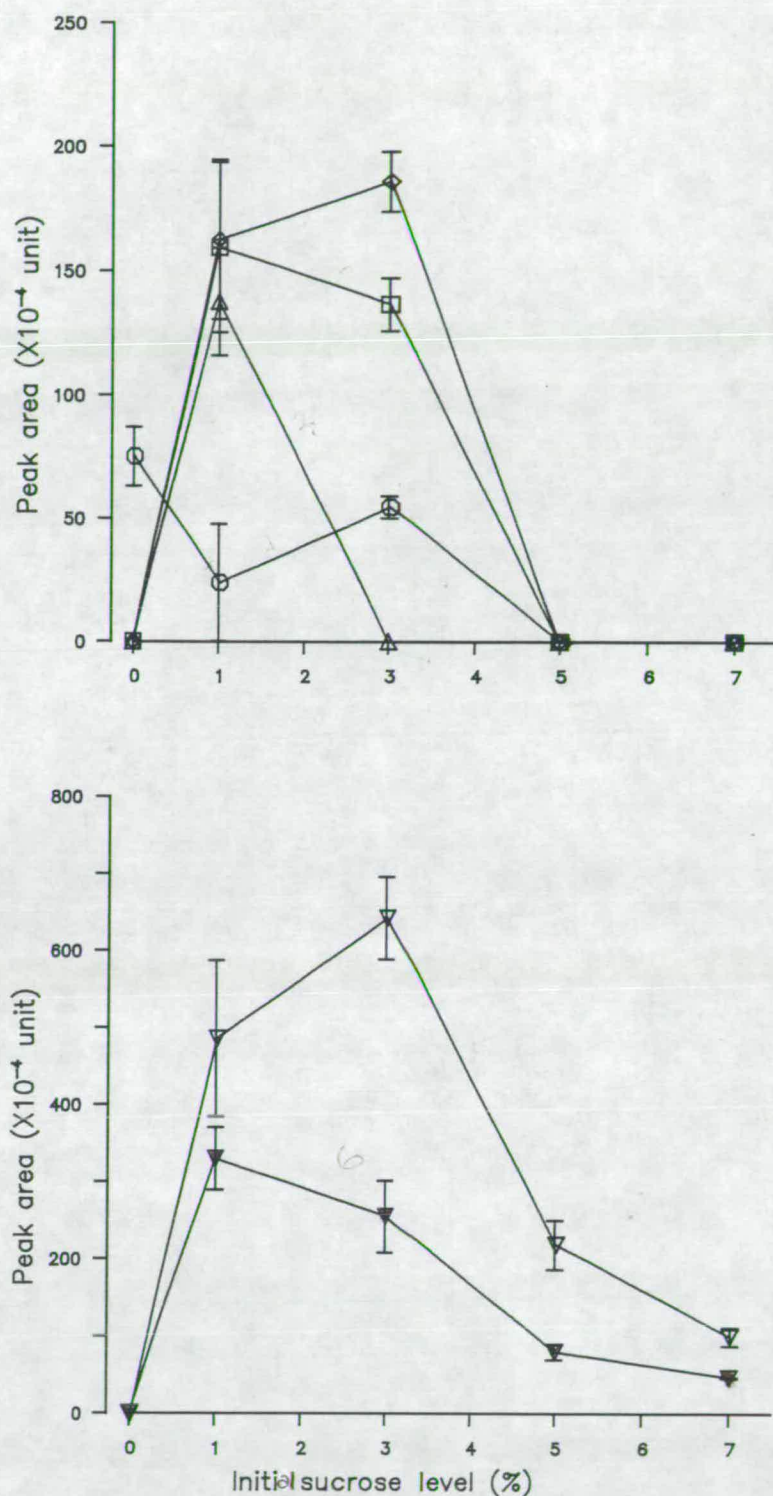


Fig. 3.1.15. The effect of sucrose on the accumulation of phenolics released into the medium of suspension cultures of *Capsicum frutescens*. Cells were in SH medium and harvested after 21d. Peak areas were obtained by injecting 20 μ l of extract [1.0 ml *per* culture] into the HPLC chromatograph. O: peak-1, Δ : peak-2, \square : peak-3, \diamond : peak-4, ∇ : peak-5 and \blacktriangledown : peak-6 as described in Fig. 3.1.10. Bars represent SE of the mean of three replicates.

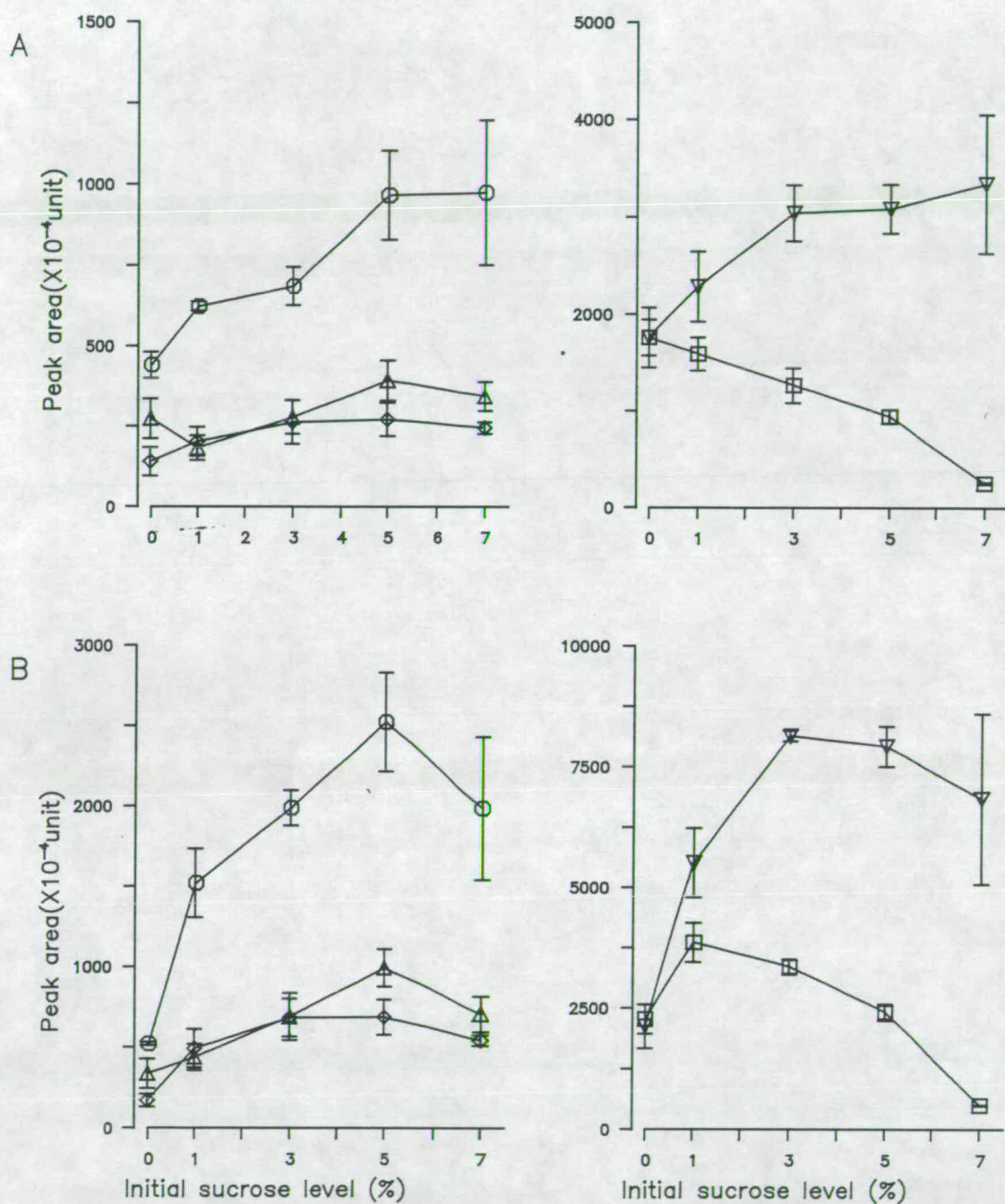


Fig. 3.1.16. The effect of sucrose on the accumulation of conjugated water soluble phenolics in suspended cells of *Capsicum frutescens*. Cells were subcultured in SH medium and harvested after 21d. Peak areas were obtained by injecting 15 μ l extracts of 1.0ml/5g(FW) cells [A] or 1.0ml/culture [B] into the HPLC chromatograph. \circ : peak-4, \diamond : peak-5, Δ : peak-6, \square : peak-7 and ∇ : peak-8 (described in Fig. 3.1.12). Bars represent SE of the mean of three replicates.

3.1.3.3. The effect of sucrose on the accumulation of phenolics in cells

The levels of phenolics-4 to 8 (see Fig. 3.1.12) were measured. The sticky materials in the extract were removed by passing the extract through a Bondelut C-18 column (Anachem International) and eluting with water. By using this method, phenolic-1 was also eluted from the column. Phenolics-2 and 3 were present at very low levels and could not be measured. The decrease in level of phenolics in the medium with the increasing concentration of sucrose coincides with the increase of most of the conjugated phenolics in the cells (see Fig. 3.1.15 and 3.1.16]. Apart from phenolic-8 there was no further increase in the levels of these phenolics with 7% sucrose. One exception was phenolic-7, synthesis of which was inhibited by the increased level of sucrose in the medium. The highest level of this phenolic was observed at 0% sucrose.

Increase in sucrose concentration appears to enhance the glycosylation of certain phenolics, but at 7%, it possibly suppresses the synthesis of phenolics. A possible explanation is that the phenolic compounds in higher plants are products of the shikimate pathway. Shikimic acid itself is derived from phosphoenol pyruvate (PEP) and erythrose-4P which are product of glycolysis and the pentose phosphate pathway respectively. Both pathways use glucose as substrate. Glucose and fructose which are the hydrolysis products of sucrose, are substrates for both pathways. Both compounds possibly have the capacity to control the enzyme activities involved in one or both pathways which will lead to a decrease in the availability of precursors for phenolic synthesis. ^{Phospho}fructokinase, one of the enzymes in glycolysis, is strongly inhibited by fructose (Turner and Turner, 1980).

3.1.4. Summary

Callus and suspension cultures accumulate significant amounts of conjugated water soluble phenolics and lignin. Free phenolics were not detected in the cells of callus and suspension cultures. In suspension cultures, free phenolics were released into the medium. The effect of growth rate and the accumulation of phenolics was very obvious in suspension cultures. Slow growing MS cultures accumulated higher

levels of free phenolics in the medium and lignin in the cells than rapidly growing SH cultures. An increase in sucrose level in the medium up to 7% (w/v) reduced the release of free phenolics into the medium and increased the level of glycosylated phenolics in the cells. The level of free phenolics in the medium and water soluble conjugated phenolics in the cells changed over the culture period. Since capsaicin was not detected in these cultures under conditions described, it is impossible to relate the accumulation of phenolics and the synthesis of capsaicin. To study these biosynthetic relationships, further studies were carried out with chilli pepper fruits.

**Section 3.2. Accumulation of Phenolics in the Developing
Fruits of *Capsicum frutescens***

3.2. Accumulation of Phenolics in Developing Fruits of *Capsicum frutescens*

It has been shown that capsaicin is synthesized only at a certain stage of fruit development in chilli pepper (Iwai *et al.*, 1979; Holden *et al.*, 1987a). In the experiments described in this section, the accumulation of free phenolics, water soluble conjugated phenolics and lignin in the developing fruit was studied in relation to capsaicin synthesis. It is well established, that capsaicin is only accumulated in placental tissue (Ohta, 1962; Balbaa *et al.*, 1968; Iwai *et al.*, 1977 and Rowland *et al.*, 1983), therefore, the compartmentation of phenolics between fruit tissues was also investigated. This experiment provides the basic information required to investigate the biosynthetic relationship between capsaicin and the other phenolics.

3.2.1. Growth of Fruits

In this experiment, fruits were gathered from plants grown in a glass house as described in 2.1. More than 100 fruits were labelled after anthesis, then every 5d., five fruits were removed and their length and FW measured. After 50d., the measurement was carried out every 10d. Fruits were then stored at -20°C until required for analysis. Fig.3.2.1.a shows the physical appearances of fruits at different stages of development. The growth curve of fruits is presented as Fig. 3.2.1.b. Fruits appeared 3d. after anthesis. Length and FW increased slowly from d.5 to d.10 then increased rapidly from d.10 to d.30. After d.30 there was no further increase in length, but the FW still increased until d.45, subsequently the FW decreased gradually from d.45 to d.70 due to the loss of water. For the purpose of these studies, fruit development is divided into three stages. Stage I in which fruits are actively elongating and capsaicin has not been produced (d.0 to d.20), stage II in which fruits have reached a maximum length and capsaicin is synthesized and accumulated (d.25 to d.40) and stage III in which the synthesis of capsaicin has ceased (after d.40).

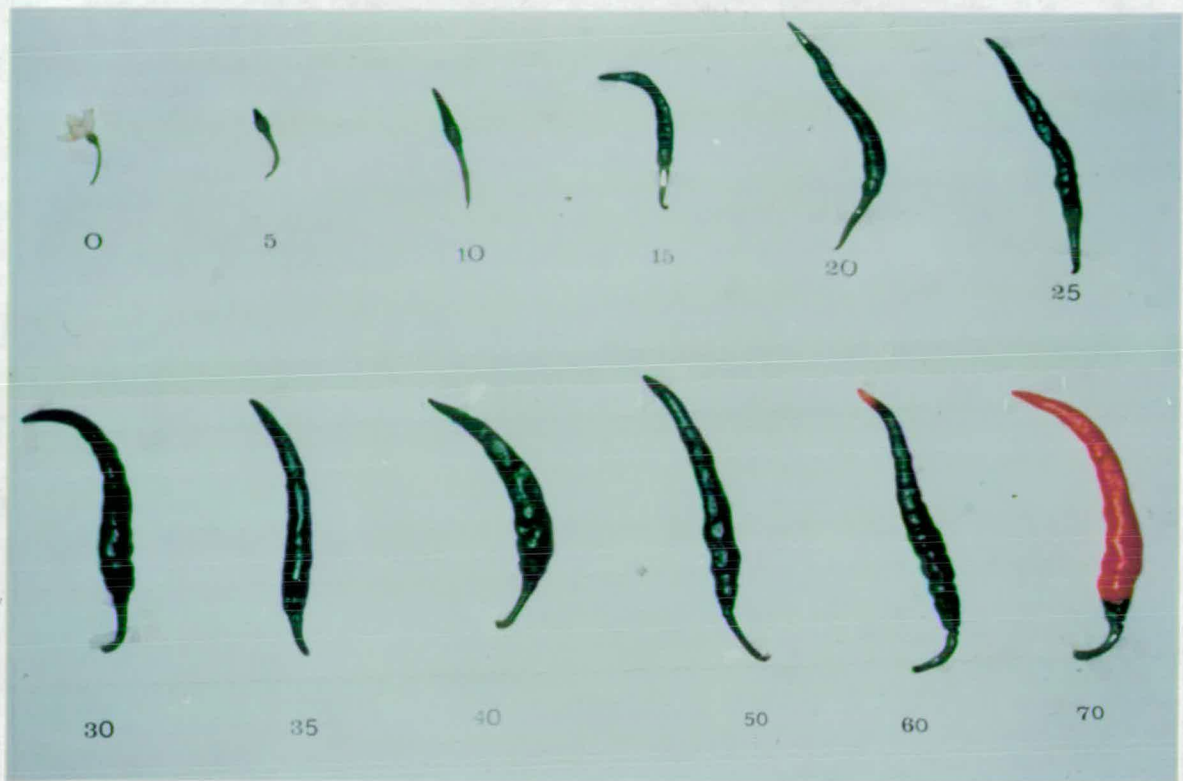


Fig. 3.2.1.a. Fruits of *Capsicum frutescens* at various developmental stages. Number= days from anthesis.

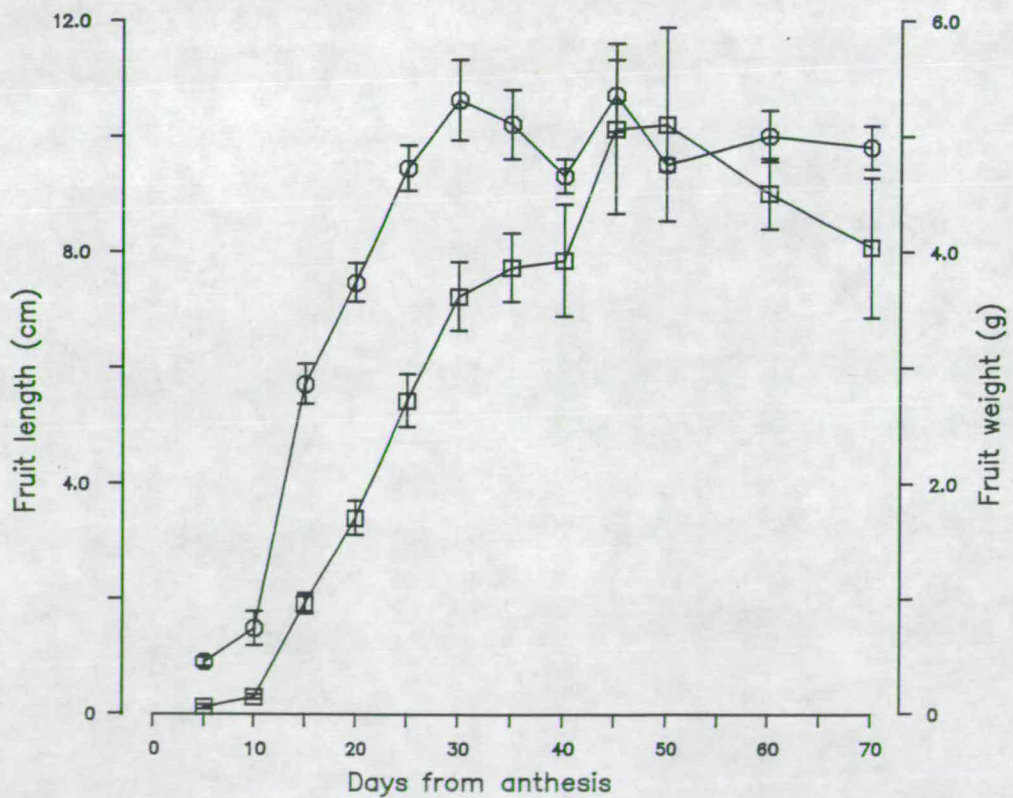


Fig. 3.2.1.b. Growth curve of fruits of *Capsicum frutescens*, fruit length [○—○], fruit FW [□—□]. Bars represent SD of the mean of three replicates

3.2.2. Identification of Phenolics in Fruits

3.2.2.1. Free phenolics

The free phenolic fraction was analysed by HPLC. following extraction and fractionation according to the method described in 2.3.2.1. Four compounds were detected in the free phenolic fraction (see Fig. 3.2.2). Peaks-1 and 2 are water soluble conjugated phenolics present in this fraction due to incomplete separation between the water and organic phase during liquid-liquid extraction. These compounds correspond with peaks-9 and 17 respectively (see their UV spectra in Fig.3.2.2). Peaks-3 and 4 are capsaicin and dihydrocapsaicin respectively.

3.2.2.2. Identification of flavonoids

Peaks-14 to 17 in Fig. 3.2.2 were identified as flavonoids from their UV spectra. Further identification of the individual flavonoids was carried out using UV spectrophotometry according to the method of Mabry (1969). The isolation of flavonoid-1 and flavonoid-2 (peaks 17 and 14 in Fig. 3.2.2) was described in 2.4.2. The UV spectrum of flavonoid-1 in MeOH shows the second λ_{\max} at 350nm (see Fig. 3.2.4), this indicates that the compound has a flavon or flavonol skeleton (see Fig. 3.2.3).

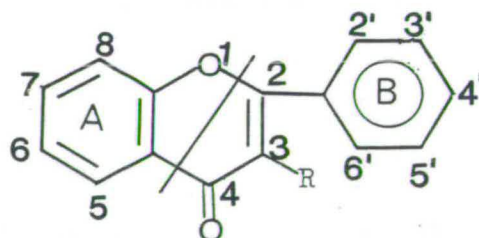


Fig. 3.2.3. The skeleton of flavon ($R=H$) and flavonol ($R=OH$)

Rings A (benzoyl chromophore) and B (cinnamoyl chromophore) are responsible for the absorbtion at 240-280nm (band II) and 300-380nm (band I) respectively [see the UV spectrum of flavonoid-1 in MeOH (Fig. 3.2.4.A)]. On the addition of NaOMe the λ_{\max} of band I increased by 50 nm (to 400 nm) without a decrease in intensity and this spectrum was stable for 10min. This indicates the presence of a free OH at 4' and

UV spectral data of peaks on Fig. 3.2.2.

Peak No	λ_{max} (nm)
1	292 318
2	252 262 350
3,4	278
5	254
6	256 293
7	287
8	295 324
10	295(sh) 308
11,12	287-305
13	256
14	252 266 350
15	260 350
16	254 350
17	252 262 350

Note: -sh: shoulder

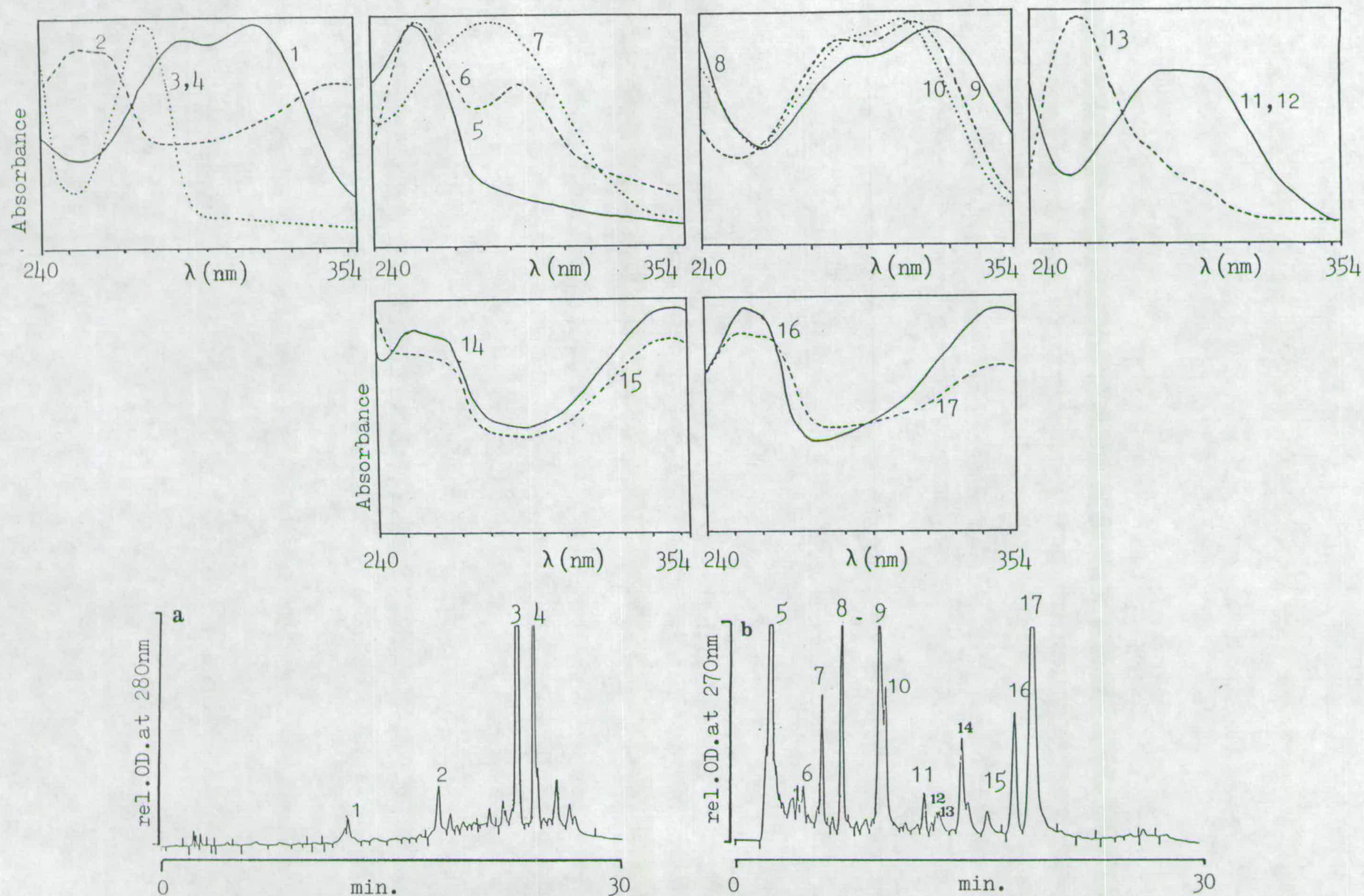


Fig. 3.2.2. HPLC chromatograms of free phenolic [a] and water soluble conjugated phenolic extracts [b] and the UV spectra of the corresponding peaks. The HPLC method was as described in 2.3.4

the absence of a free OH at the 3 position. Sodium acetate (NaOAc) is a weaker alkali than NaOMe and is useful in diagnosing the presence of a free OH group at the 7 position. The λ_{max} of band II increased by 8nm on the addition of NaOAc and the spectrum was stable for 10min. This indicates the presence of a free OH at position 7. After the addition of boric acid, the λ_{max} of band I was still 15nm higher than a solution in MeOH. This indicates the presence of an ortho dihydroxyl in ring B. The presence of these functional groups was also shown by the fact that an unstable complex was formed between the compound and AlCl_3 in the presence of HCl. Aluminium chloride formed a stable complex with the carbonyl group at position 4 and an OH group at position 3 or 5. In addition to the unstable complex produced between AlCl_3 and the ortho dihydroxyl groups in ring B, the compound and the reagent also formed a stable complex. This was shown by the presence of a double peak in band I, with a longer maximum wave length still higher than the λ_{max} of band I in MeOH (see the UV spectrum of flavonoid-1 in AlCl_3 and HCl in Fig. 3.2.4.A). Since the free OH group at position 3 is absent, this complex must be formed between AlCl_3 with a carbonyl group at position 4 and an OH group at the 5 position. The compound is possibly 3-glycosyl-3',4',5,7-tetrahydroxyflavon. On acid hydrolysis rhamnose and quercetin were released (see Fig. 3.2.4.B and C). Therefore the probable structure of this compound is 3-rhamnosyl-3',4',5,7-tetrahydroxyflavonol (3-rhamnosylquercetin).

The UV spectrum of flavonoid-2 in MeOH is similar to flavonoid-1 with a lower intensity in band II (see Fig. 3.2.5.a). The response of this flavonoid to the addition of NaOMe, boric acid and AlCl_3 was the same as flavonoid-1. The only difference observed was that NaOAc did not increase the λ_{max} of band II. This indicates the absence of a free OH group at the 7 position. There are two possible structures for this compound: 1). 3,7-diglycosyl-3',4',5-trihydroxyflavonol (3,7-diglycosyl quercetin) or 2). 7-glycosyl-5,3',4'-trihydroxyflavone (7-glycosylluteolin). A comparison of the acid hydrolysis products of flavonoid-2 with authentic standards on HPLC, led to the identification of luteolin and glucose. Therefore the proposed structure of flavonoid-2 is 7-glucosylluteolin.

Fig. 3.2.4.

Identification of flavonoid-1. A). The UV spectrum of flavonoid-1 in MeOH and after addition of the diagnostic reagents. B). The HPLC chromatogram of sugar released by flavonoid-1 following acid hydrolysis (gly.1) and the cochromatogram with rhamnose(Rha). The HPLC method was as described in 2.4.5. C). The HPLC chromatogram of the aglycone released by flavonoid-1 following acid hydrolysis (Fl.1) and the cochromatogram with quercetin (Quer). The HPLC method was as described in 2.3.4

UV spectral data of flavonoid-1

Solvent	$\lambda_{\max}(\text{nm})$					
MeOH	256	260	268			350
MeOH+NaOMe		262(sh)	268		327(sh)	400
MeOH+NaOAc		262(sh)	268		320(sh)	390
MeOH+NaOAc+H ₃ BO ₃	262(sh)	268				365
MeOH+AlCl ₃		262(sh)	268	274(sh)	304(sh)	426
MeOH+AlCl ₃ +HCl		261	268	298(sh)		358 395

Note: - sh: shoulder

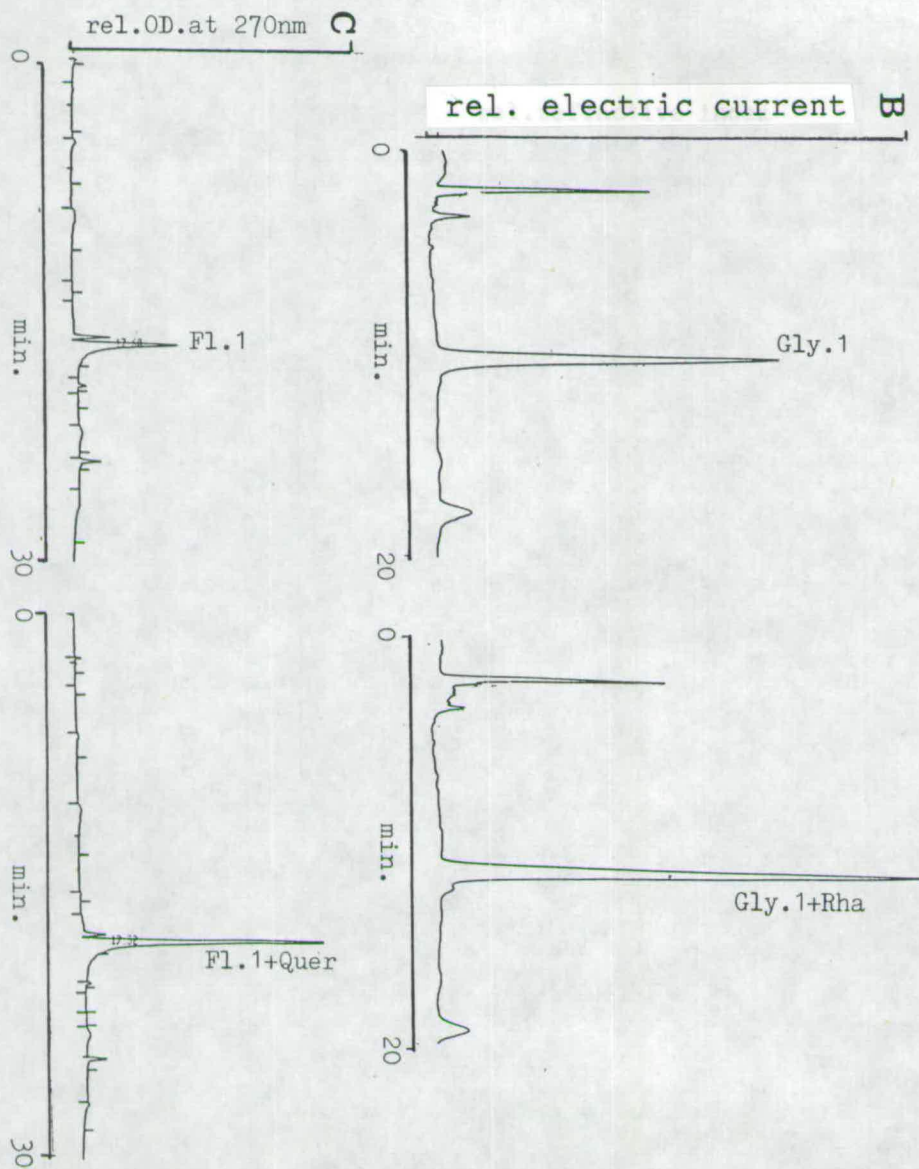
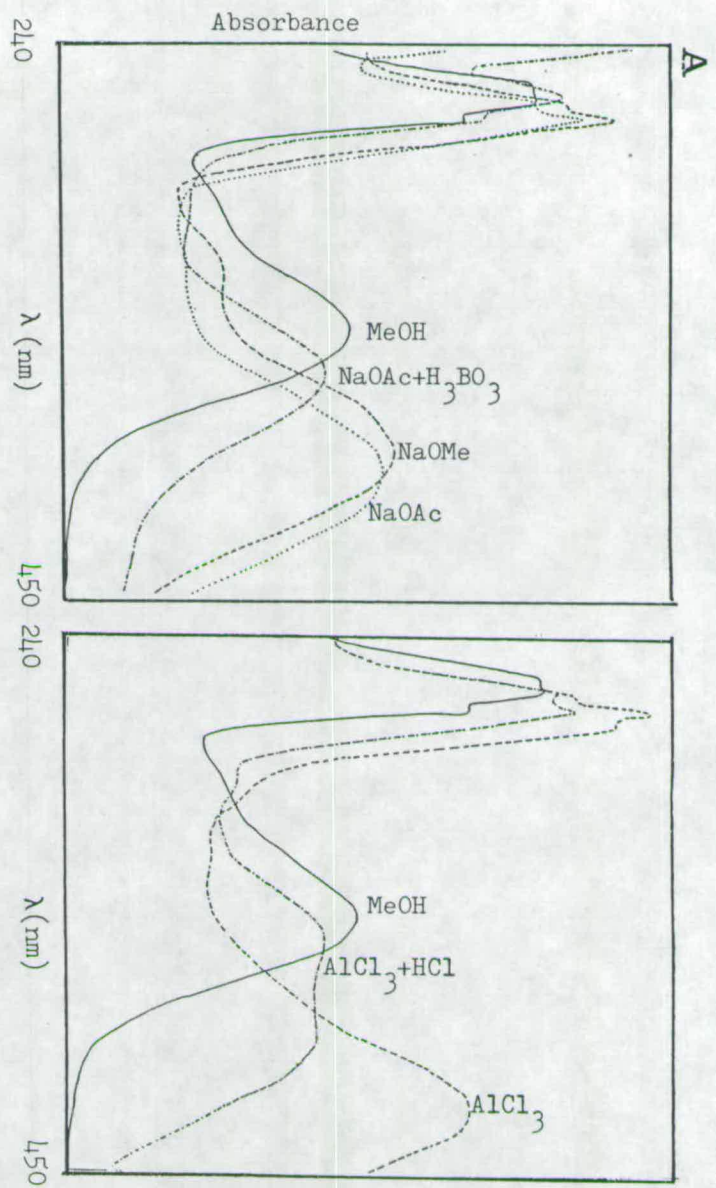


Fig. 3.2.4.

Using the same method of analysis, the presence of free ortho dihydroxy groups at the 3' and 4' positions and the absence of free hydroxyls at the 3 and 7 positions in flavonoid-3 (peak-16 in Fig. 3.2.3) were ascertained. The UV spectrum of this compound is not the same as 3,7-diglycosylquercetin presented in Mabry, (1969) and there was no obvious stable complex formed between this compound and AlCl_3 in the presence of HCl. Since the amount of the isolate obtained was only small, further identification was not possible. Flavonoid-4 (peak 14 in Fig. 3.2.3) was present at a much lower level in the fruit than the other flavonoids and was not isolated. From its UV spectrum, it seems that this phenolic also has a flavon or flavonol skeleton.

3.2.2.3. Identification of water soluble conjugated hydroxycinnamates

Peaks 8, 9 and 10 (see Fig. 3.2.3) can be identified as conjugated hydroxycinnamic acids from their UV spectra. Further identification was carried out by labelling the compounds with $[^{14}\text{C}]$ cinnamic acid. The method for the isolation and identification of these compounds was described in 2.4.4. Since only small quantities of the compounds were isolated, it was difficult to identify the non-radioactive sugar moieties bound to these phenolics.

i. Peak-8

The UV spectrum of this compound is typical of 3,4-dioxycinnamates (see appendix). On acid and alkaline hydrolysis, this compound was degraded. On incubation with emulsin, this compound released caffeic acid (3,4-dihydroxycinnamic acid) which was identified by TLC followed by RITA scanning and comparison with an authentic standard. Therefore this compound is tentatively identified as caffeoyl glycoside.

ii. Peak-9

The UV spectrum of this compound is also typical of 3,4-dioxycinnamates. On acid and alkaline hydrolysis this substance released a compound which coeluted with cinnamic acid, and a degradation product. On incubation with emulsin, only one

Fig. 3.2.5.

Identification of flavonoid-2. A). The UV spectrum of flavonoid-2 in MeOH and after addition of the diagnostic reagents. B). The HPLC chromatogram of sugar released by flavonoid-2 following acid hydrolysis (gly.2) and the cochromatogram with galactose (Gal) and glucose (Glu). The HPLC method was as described in 2.4.5. C). The HPLC chromatogram of the aglycone released by flavonoid-2 following acid hydrolysis (Fl.2) and the cochromatogram with luteolin (Lut). The HPLC method was as described in 2.3.4

UV spectral data of flavonoid-2

Solvent	$\lambda_{\max}(\text{nm})$			
MeOH	257(sh)	268		352
MeOH+NaOMe		267	282(sh) 298(sh)	394
MeOH+NaOAc		267	282(sh)	414
MeOH+NaOAc+H ₃ BO ₃	258		282	372
MeOH+AlCl ₃		274	298(sh)	326 425
MeOH+AlCl ₃ +HCl		274	295	360 384

Note: - sh: shoulder

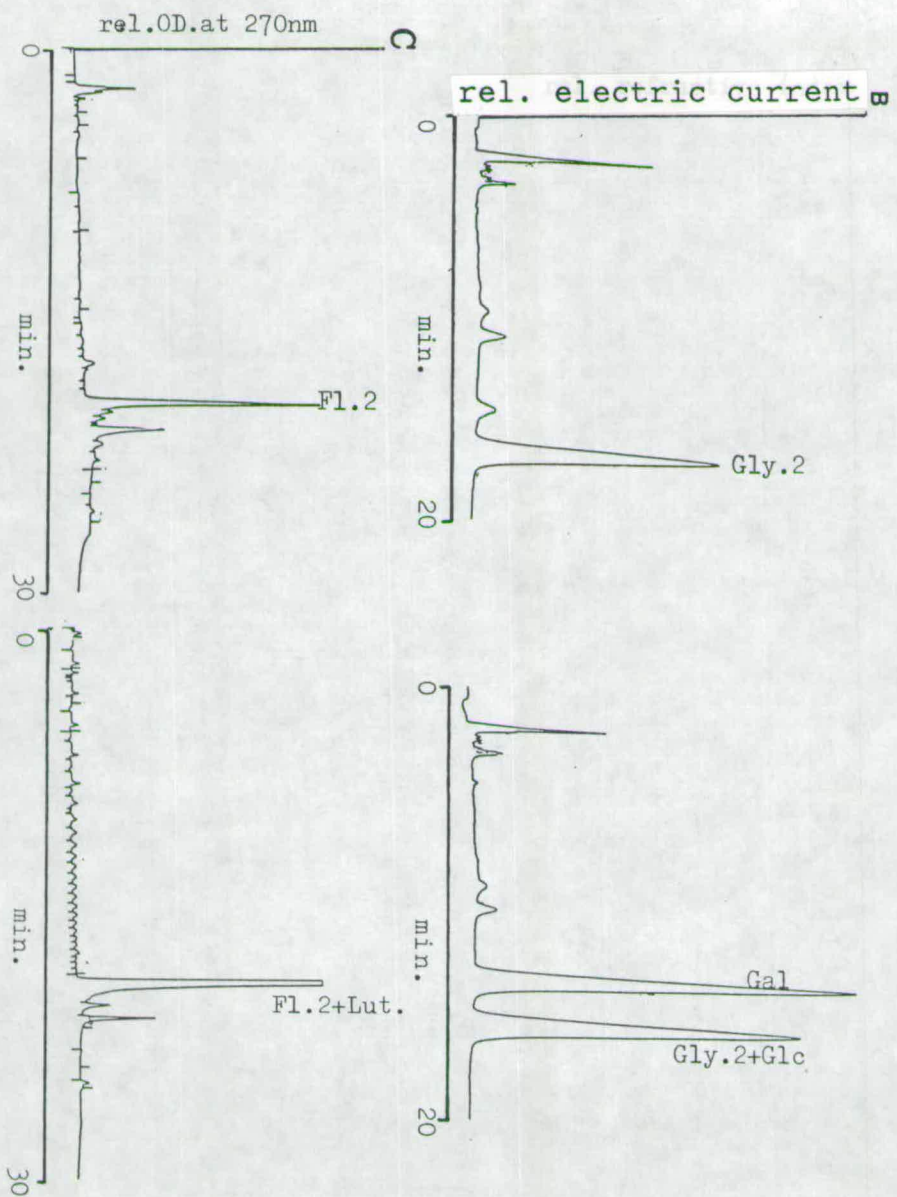
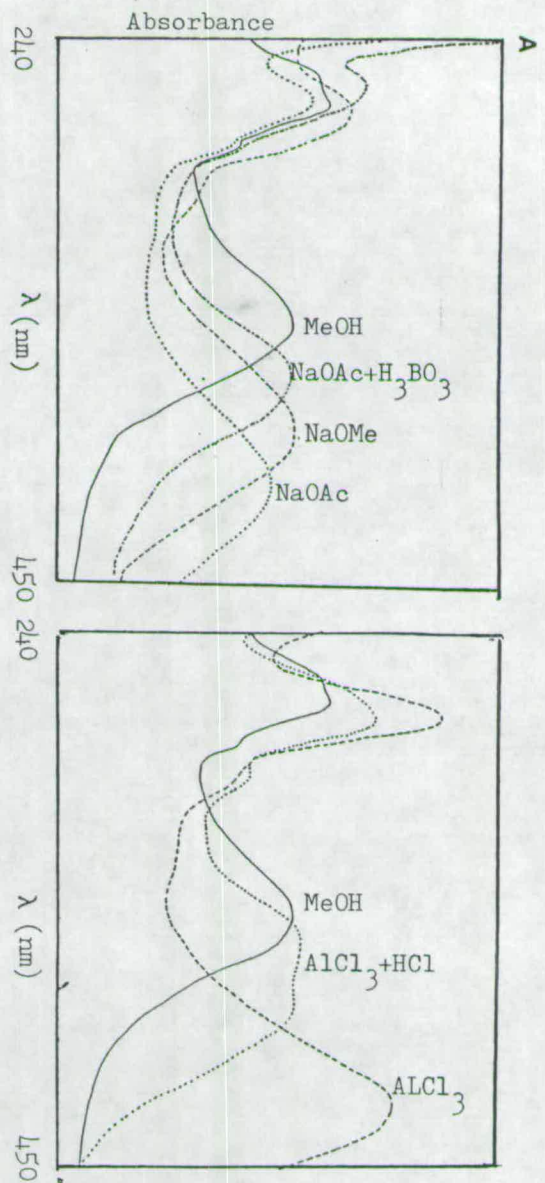


Fig. 3.2.5.

compound which coeluted with cinnamic acid on TLC was produced. This indicates that the phenolic is bound to a sugar via an ester bond. The free phenolic released has higher R_f and R_t values than ferulic acid (3-methoxy-4-hydroxycinnamic acid) in the TLC and HPLC systems used. This indicates that this compound is less polar than ferulic acid. The decrease in polarity occurs when the free hydroxyl group is acylated or alkylated. The possibility of the compound being acylated can be ruled out, since the ester would be hydrolysed with alkali. A methylated ferulic acid was finally prepared by methylation of ferulic acid with dimethylsulphate and the product analysed by HPLC. Indeed, the 3,4-dimethoxycinnamic acid obtained has an R_t value close to cinnamic acid. Therefore compound of peak 8 is tentatively identified as 3,4-dimethoxycinnamoyl glycoside.

iii. Peak-10

The UV spectrum of this compound is typical of p-oxy coumarates (see appendix). On acid hydrolysis, the compound was degraded. On alkaline hydrolysis, the compound released p-coumaric acid and a degradation product. On incubation with emulsin, only p-coumaric acid was released. Therefore this compound is tentatively identified as p-coumaroyl glycoside.

Although emulsin (β -glucosidase from almond) released the phenolic moieties from the bound forms, the sugar moiety is possibly not glucose. There is some evidence to support this possibility. Based on the size of peaks produced on RITA scanning, it seems that the amount of phenolics released on enzymic hydrolysis were much lower than the original compounds. It has been shown that emulsin (β -glucosidase from almond) also hydrolyses non- β -glucosides such as α -D-galactoside, β -D-xyloside, β -L-arabinoside (Hestrin *et al.*, 1955). The p-coumaroyl glycoside found in fruits has a different R_t value from the synthesized p-coumaroyl- β -D-glucose in the HPLC system used.

3.2.2.4. Identification of the other phenolics

Peak-5 was possibly due to a mixture of phenolics which were not retained by the column. The low R_t value indicates that the compound is very polar which is possibly due to intensive glycosylation. So far, there is not enough information to speculate about the identity of the phenolic moiety of this compound. The phenolics of peaks-6 and 7 (see Fig. 3.2.3) were tentatively identified as glycosides of vanillic acid and p-hydroxybenzaldehyde respectively from their UV spectra. None of the UV spectra of the phenolics listed in appendix has a UV spectrum similar to those of the compounds of peaks-11, 12 and 13 (see Fig. 3.2.3). Although a small amount of the compound of peak-13 has been isolated, its identity is still unknown. This compound gave a blue colour on TLC after spraying with 10% (v/v) H_2SO_4 in MeOH followed by heating at $105^\circ C$ for 10min. With the diazo reagent, it gave a red colour. It is obvious that this compound has an OH phenolic group. The tentative identity of all the phenolics detected in fruits of *Capsicum frutescens* is summarized in Table 3.2.1.

3.2.3. Accumulation of Phenolics

In this experiment at least 5 fruits were extracted and fractionated according to the methods described in 2.3.2.1. The free and the water soluble conjugated phenolic extracts were dissolved in 1.0 ml of MeOH and 80% MeOH respectively *per* 2.0g(FW) and 10 μ l samples were injected onto the HPLC chromatograph. The amount of phenolic *per* fruit was calculated from the amount *per* g(FW) and the average FW.

3.2.3.1. The accumulation of capsaicin (see Fig. 3.2.6)

Capsaicin in fruits of *Capsicum frutescens* was first detected 25d. from anthesis, when the increase in fruit length had ceased (see Fig. 3.2.1.b). Subsequently the level of capsaicin increased rapidly until d.40. However, there was a small decrease in capsaicin level *per* g(FW) between d.40 and d.50. At this stage, the synthesis of capsaicin has possibly stopped but the FW was still increasing. From d.50 to d.70 the

Table 3.2.1. The proposed identitiy of phenolics in chilli pepper fruits

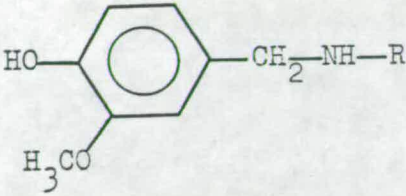
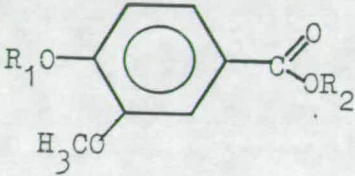
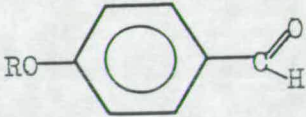
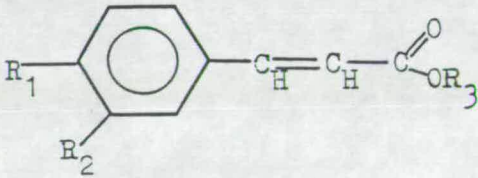
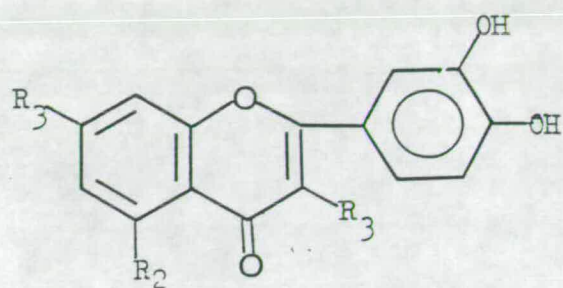
Structure	Peak No	Substituent	Compound
	3	$R=C_{10}H_{17}O$	Capsaicin
	4	$R=C_{10}H_{19}O$	Dihydro capsaicin
unknown	5		unknown
		$R_1=R_2:$ H/Sugar	Vanillic Glycoside
	7	$R=\text{Sugar}$	p-Glycosido benzaldehyde
	8	$R_1=R_2=OH$ $R_3=\text{Sugar}$	Caffeoyl glycoside
	9	$R_1=R_2=OCH_3$ $R_3=\text{Sugar}$	3,4-Dimethoxy cinnamoyl glycoside
	10	$R_1=OH$ $R_2=H$ $R_3=\text{Sugar}$	p-Coumaroyl glycoside

Table 3.2.1. (Continued)



Structure	Peak No	Substituent	Compound
unknown	11		unknown
unknown	12		unknown
unknown	13		unknown
	14	$R_1=H$ $R_2=OH$ $R_3=Glucose$	7-Glucosyl luteolin
	15	R_1, R_2 and $R_3=unknown$	Flavonoid-4
	16	R_1, R_2 and $R_3 = unknown$	Flavonoid-3
	17	$R_1=O-Rha$ $R_2=R_3=OH$	3-Rhamnosyl quercetin

level of capsaicin *per g*(FW) increased, possibly due to the loss of water from the ripening fruits. After reaching a maximum level at d.40, the amount of capsaicin *per fruit* did not decrease. Fluctuation in the level of capsaicin *per fruit* between d.40 and d.70 is possibly due to the variation in the level in the individual fruits.

3.2.3.2. The accumulation of flavonoids (Fig. 3.2.7)

All the four flavonoids detected were present at high levels at stage I. The levels of these compounds *per g*(FW) dropped when the fruits started accumulating capsaicin (d.20 to d.25). Except for flavonoid-3, the levels *per g*(FW) either decreased or remained constant after d. 25. Flavonoid-3 was still actively accumulated until d.35 after which the level decreased until d.60. The amount of flavonoid *per fruit* also fluctuated during development. A decrease in the amounts of 7-glucosylluteolin and 3-rhamnosylquercetin was noted between d.20 and d.25. The amount of flavonoid-3 decreased after d.35. Interconversion between flavonoids or conversion of a flavonoid into another secondary metabolite may take place in fruits. A change in the flux of common intermediates may also take place in the early part of stage II leading to a decrease in flavonoid synthesis and an increase the production of capsaicin.

3.2.3.3. The accumulation of hydroxycinnamoyl glycosides (Fig.3.2.8)

The amounts of the three hydroxycinnamoyl glycosides were at their highest level when the fruits started accumulating capsaicin (d.25). The levels then decreased sharply when the fruits were actively accumulating capsaicin. *p*-Coumaroyl and caffeoyl glycosides were not detectable after d.30 and d.40 respectively. Although the amount of 3,4-dimethoxycinnamoyl glycoside was much lower in fruits actively accumulating capsaicin, it was always detectable. During fruit ripening (d.60 to d.70) the level of this compound increased.

3.2.3.4. Accumulation of the other phenolics (Fig. 3.2.9)

The pattern of accumulation of the phenolics of peaks-5 and 13 was the same as

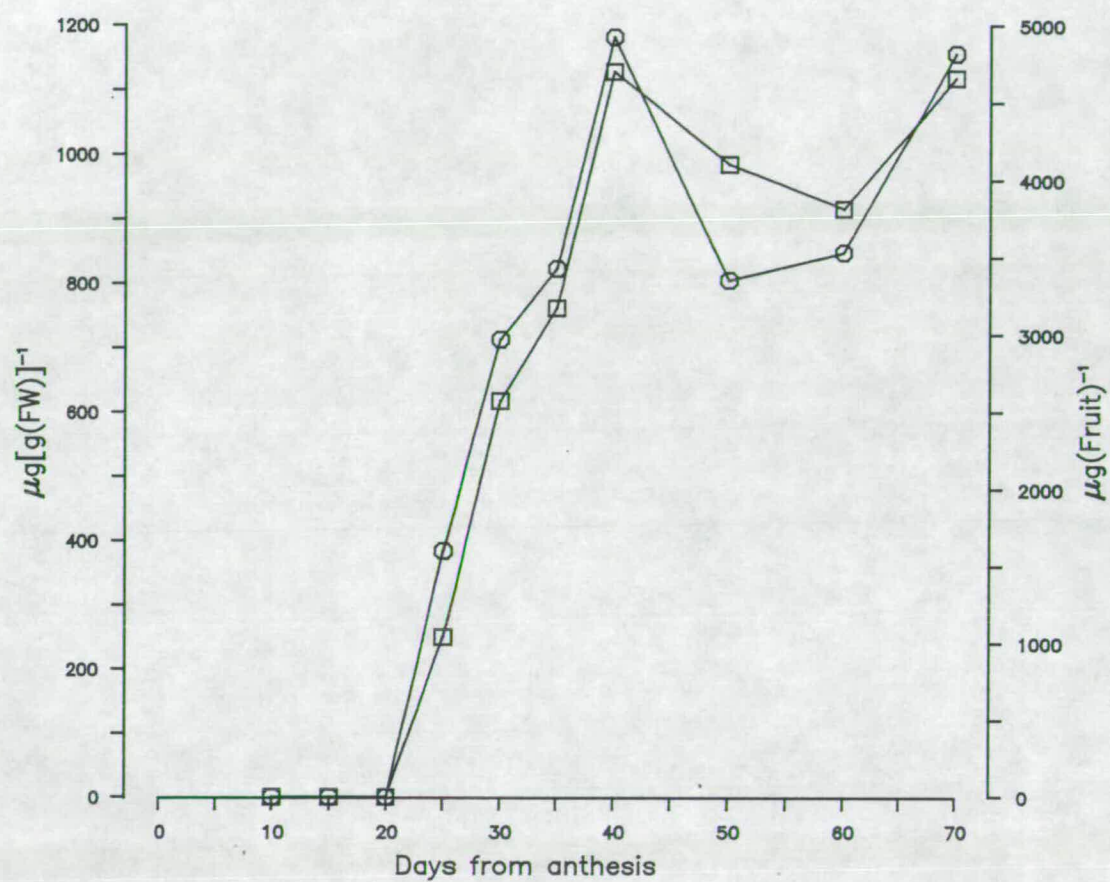


Fig. 3.2.6. The accumulation of capsaicin in fruits of *Capsicum frutescens*, per g(FW) [○ — ○] and per fruit [□ — □]

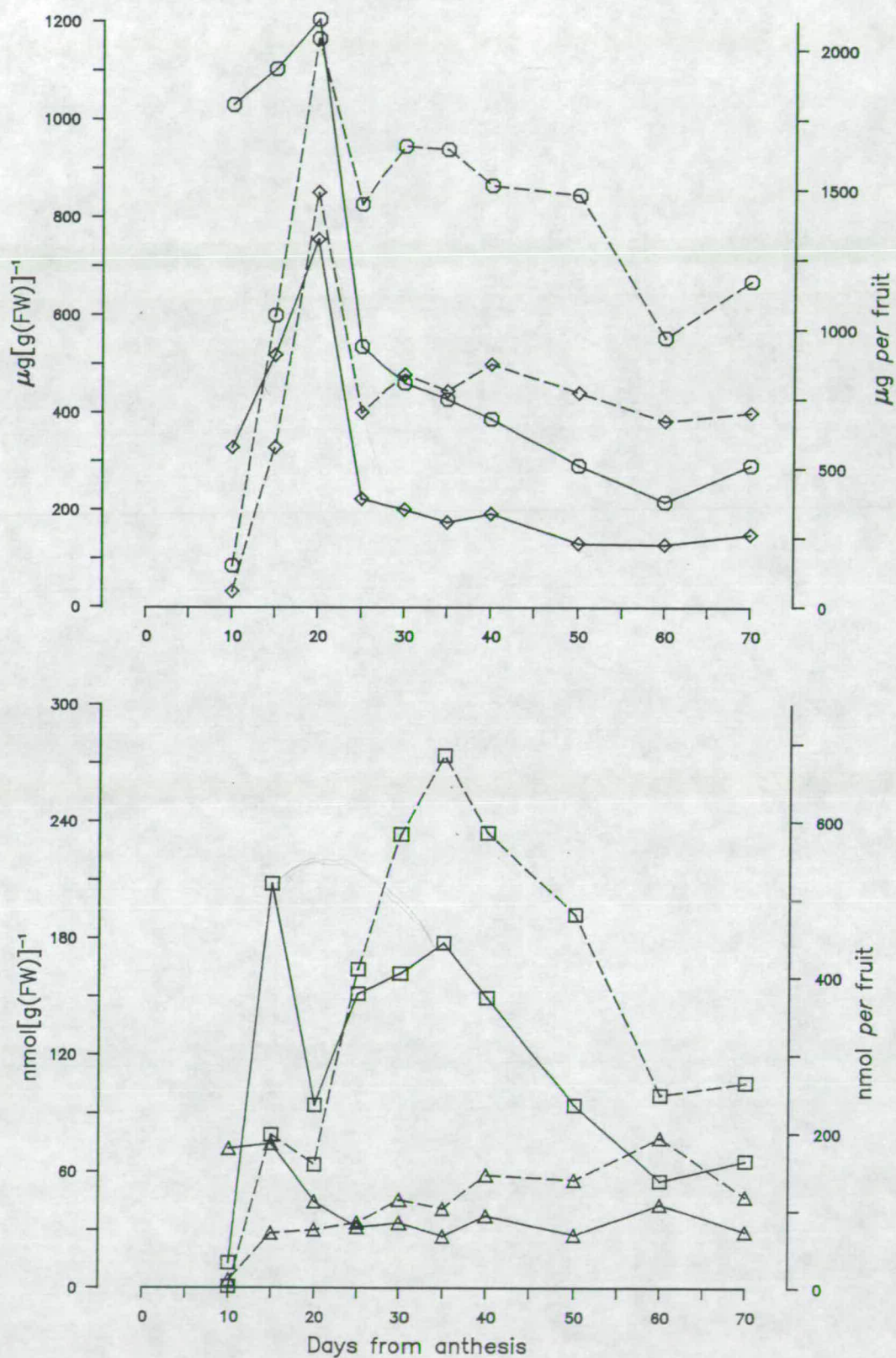


Fig. 3.2.7. Accumulation of flavonoids in fruits of *Capsicum frutescens*, 3-rhamnosylquercetin [O], 7-glucosylluteolin [◇], flavonoid-3 [□], flavonoid-4 [△], per g(FW) [—] and per fruit [----]

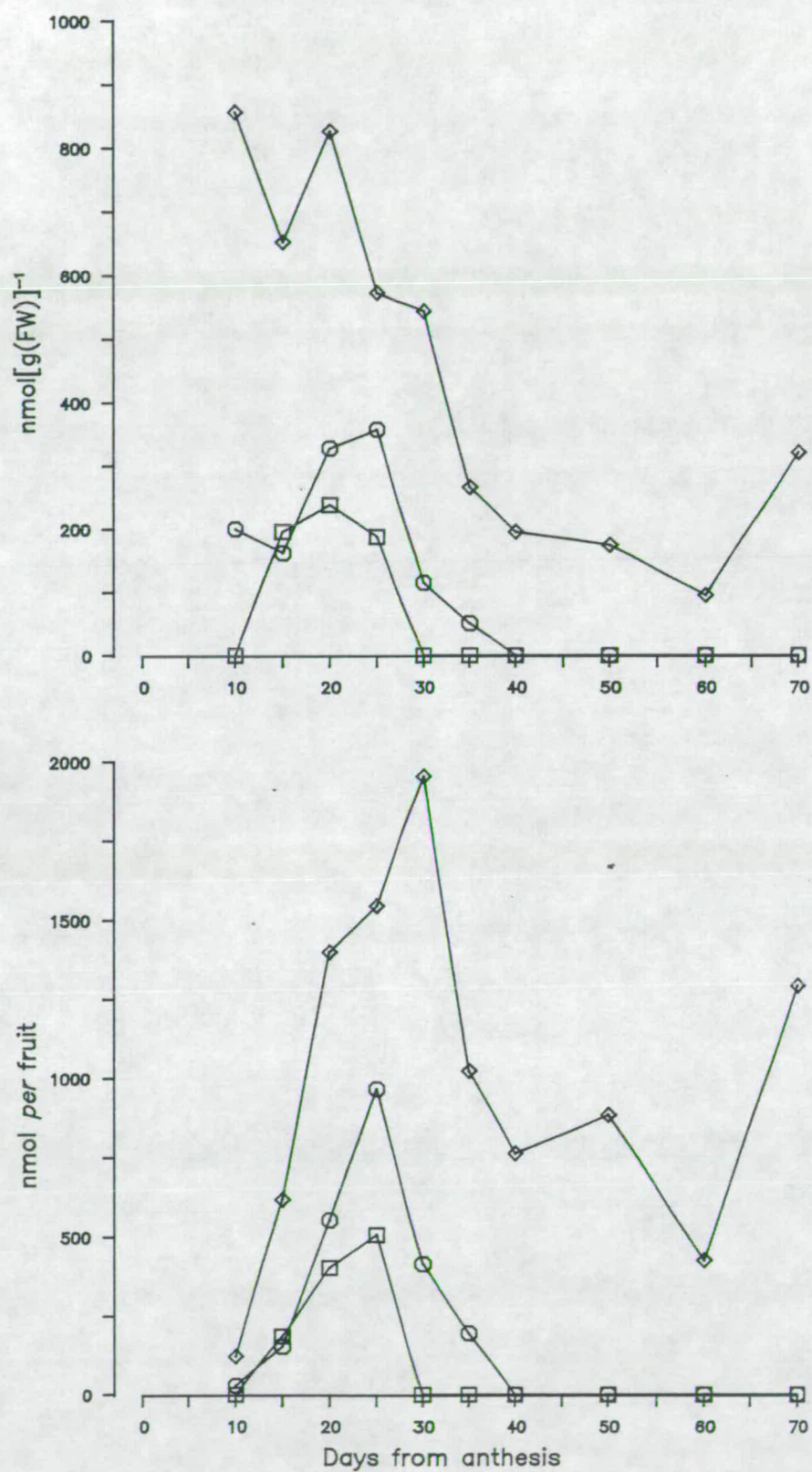


Fig. 3.2.8. Accumulation of hydroxycinnamoyl glycosides in fruits of *Capsicum frutescens*, p-coumaroyl glycoside [□], caffeoyl glycoside [○], and 3,4-dimethoxycinnamoyl glycoside [◇]

for capsaicin. These compounds were actively accumulated after d.25 and appeared not to be further metabolized (see Fig. 3.2.9 a and f). The amount of the phenolic of peak-5 continually increased during fruit development. This compound may act as an acceptor of the degradation product of the other phenolics. Vanillic acid glycoside (Fig. 3.2.9.b) was present at a very low level, and the amount *per g*(FW) increased from d.10 to d.20 then decreased when fruits started accumulating capsaicin. When the accumulation of capsaicin had ceased, the level increased again. Although the amount *per g*(FW) fluctuated during development, the amount *per fruit* increased until d.50. The level of this compound then decreased during continued fruit ripening (d.60 to d.70). The levels of p-glycosidobenzaldehyde increased markedly from d.10 to d.40, then decreased during fruit ripening. Except at d.15, the phenolic of peak-11 (see Fig. 3.2.9.d) was present at a low level during the whole of fruit development. The amount *per fruit* increased until d.40 then remained constant. The phenolic of peak-12 (Fig. 3.2.9.e) was present at a high level at d.10 then decreased sharply between d.10 and d.20 and disappeared at d.50. Between d.10 and 15 the amount *per fruit* still increased although the amount *per g*(FW) decreased. This compound is possibly an active intermediate which is actively turned over in fruits of stage I.

3.2.3.5. The accumulation of lignin (Fig. 3.2.10)

Lignin in fruits is likely to be an end product of metabolism, the decrease in lignin *per g*(FW) between d.10 and d.25 is possibly due to the increase in FW proceeding more quickly than the synthesis of lignin. The pattern of lignin accumulation was the same as for capsaicin. Lignin was actively accumulated during stage-II and there was no further increase during fruit ripening.

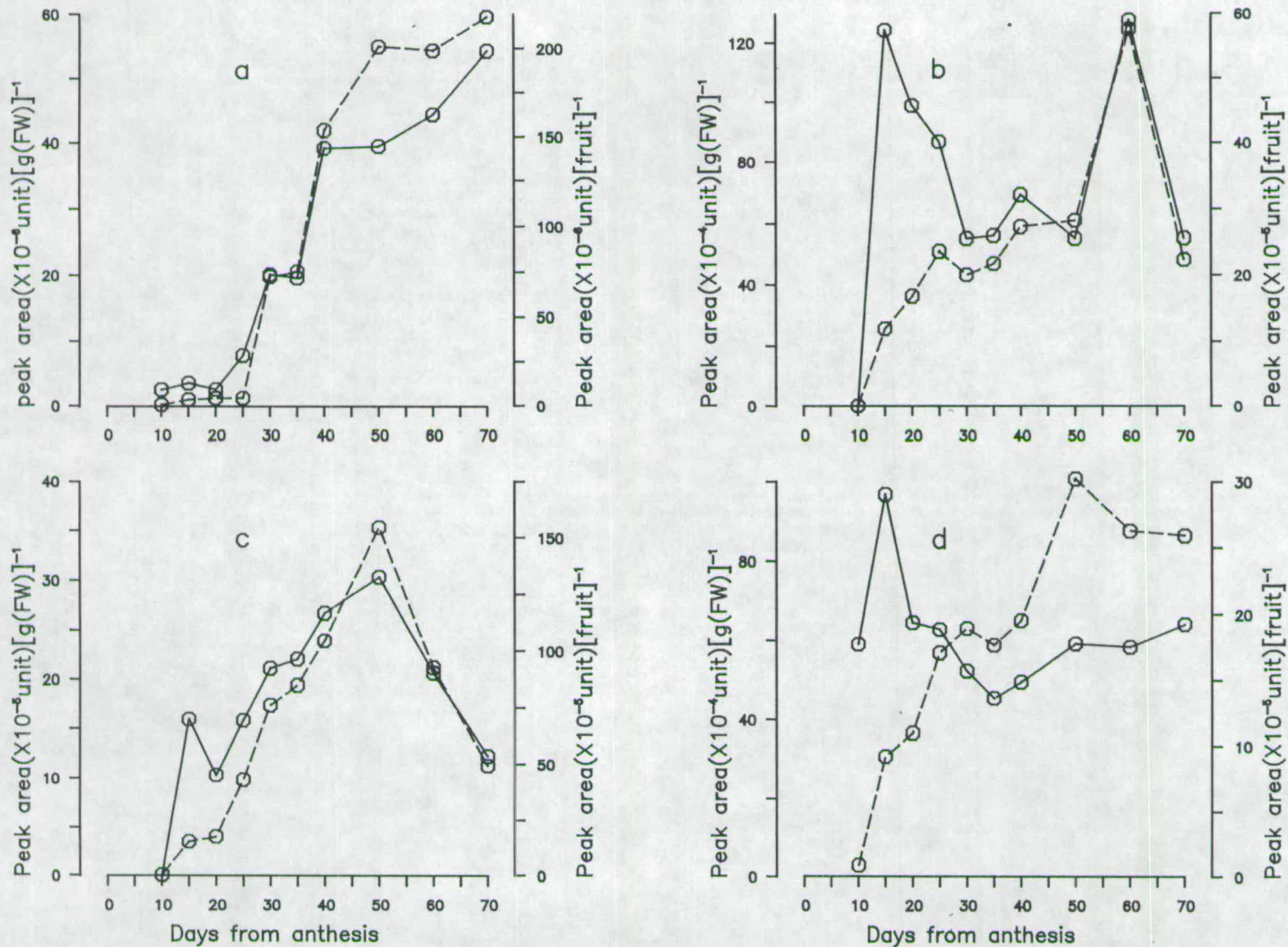


Fig. 3.2.9. Accumulation of the phenolics of peak-5 [a], vanillic glycoside [b], p-glycosidobenzaldehyde [c] the phenolic of peak-11 [d], the phenolic of peak-12 [e] and the phenolic of peak-13 [f] in fruits of *Capsicum frutescens*. [—] per g(FW) and [----] per fruit.

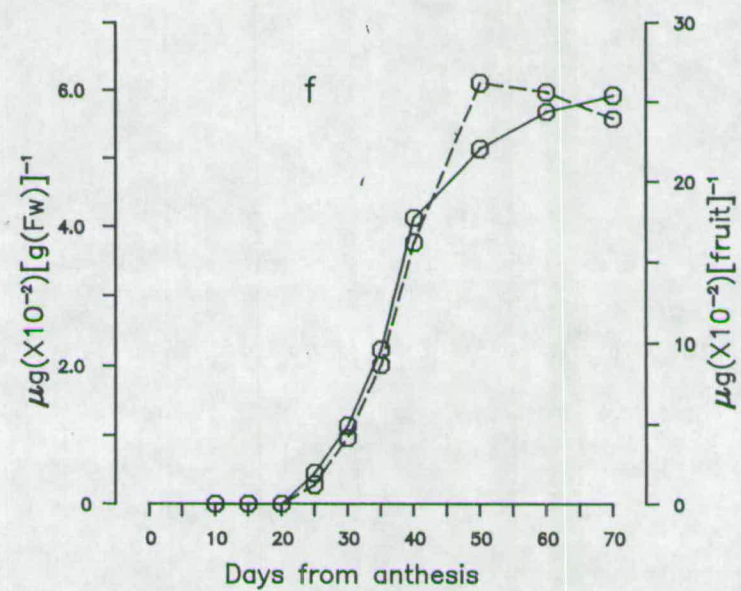
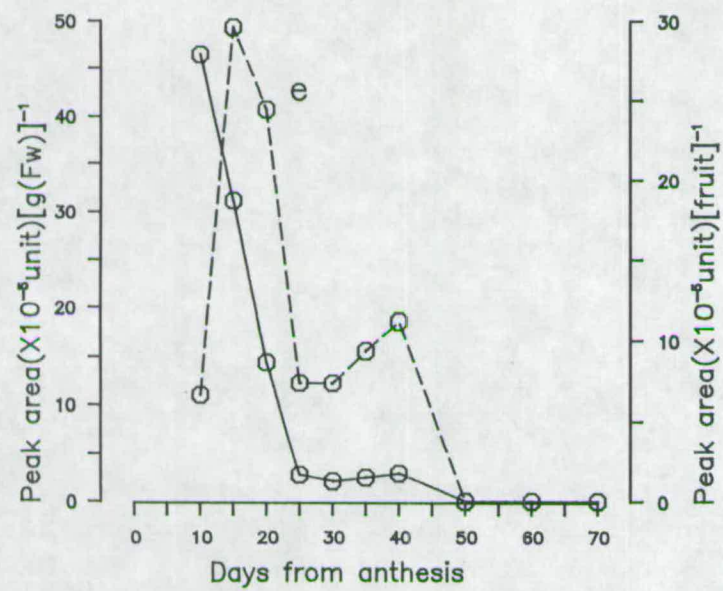


Fig. 3.2.9. (Continued)

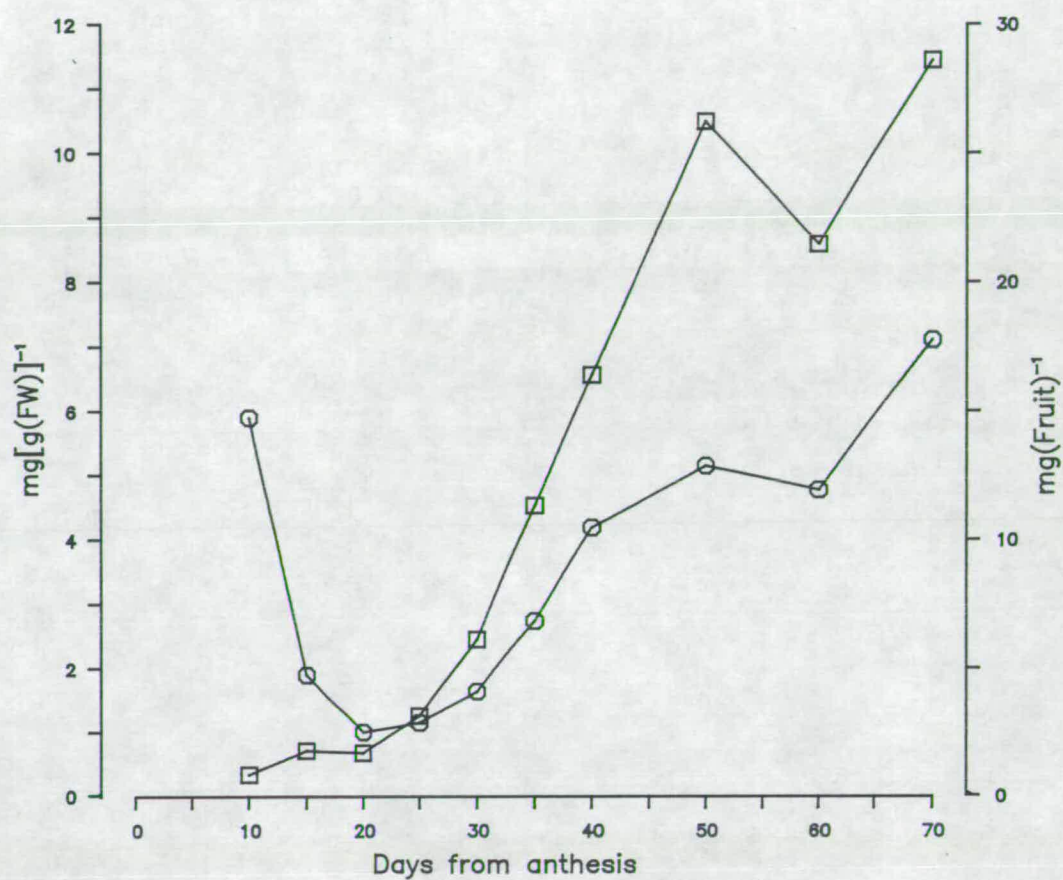


Fig. 3.2.10. The accumulation of lignin in fruits of *Capsicum frutescens*, per g(FW) [○ —○] and per fruit [□ —□]

3.2.4. Phenolic Compartmentation

It is probable that groups of phenolics may be stored in different tissues, in the chilli pepper. To investigate this compartmentation, fruits of stage II (d.35) were separated into pericarp, placenta and seed. Unfortunately, it was not possible to separate fruits of stage I (d.20) into three components, so seed and placenta were left together.

Table 3.2.2 and 3.2.3 show that flavonoids are found only in the pericarp. p-Coumaroyl and caffeoyl glycoside in fruits of stage I are found in the mixed tissues of placenta and seed, and in fruits of stage II mainly in the seed. 3,4-Dimethoxycinnamoyl glycoside is also present mainly in seed, but it is also present at significant levels in the other tissues. It is important to note that capsaicin was detected in all components of the stage II fruit. This compound is possibly synthesized in the cells of the placenta which then contaminate the other tissues. Compounds of peak-5 and p-glycosidobenzaldehyde are present mainly in the pericarp. Vanillic acid glycoside and the compound of peak-13 were only detected in the placenta and seed respectively.

3.2.5. Summary

There were no detectable free phenolics other than capsaicin in fruits of *Capsicum frutescens*. Water soluble conjugated phenolics were present in significant quantities and appeared to be turned over. Glycosylated flavonoids and hydroxycinnamates were present at high levels in stage I and decreased or disappeared completely in the later stages. The hydroxycinnamoyl glycosides which were present mainly in the placenta and seed are possibly involved in the biosynthesis of capsaicin and lignin. There may be a change in the flux of intermediates which brings about the increase in capsaicin synthesis and the decrease in production of water soluble conjugated phenolics. These possibilities will now be tested in the subsequent experiments.

Table 3.2.2. The compartmentation of phenolics in 20d. old fruits

Compound	%in Seed and Placenta	%in Pericarp
p-Coumaroyl glycoside	100.0	0.0
Caffeoyl glycoside	94.0	6.0
3,4-Dimethoxycinnamoyl glycoside	74.0	26.0
3-Rhamnosylquercetin	0.0	100.0
7-Glucosylluteolin	0.0	100.0
Flavonoid-3	0.0	100.0
Flavonoid-4	0.0	100.0
p-Glycosidobenzaldehyde	0.0	100.0
Phenolic of peak-5	11.4	88.6

Table 3.2.2. The compartmentation of phenolics in 35d.old fruits

Compound	%in seed	%in placenta	%in pericarp
Capsaicin	7.6	75.9	16.5
p-coumaroyl glycoside	100.0	0.0	0.0
Caffeoyl glycoside	89.3	10.7	0.0
3,4-Dimethoxycinnamoyl glycoside	81.9	10.0	8.1
3-Rhamnosylquercetin	0.0	0.0	100.0
7-Glucosylluteolin	0.0	0.0	100.0
Flavonoid-3	0.0	0.0	100.0
Flavonoid-4	0.0	0.0	100.0
Vanillic ^{acid} glycoside	0.0	100.0	0.0
p-Glycosidobenzaldehyde	0.0	0.0	100.0
Phenolic of peak-5	6.8	11.0	82.2
Phenolic of peak-13	100	0.0	0.0

**Section 3.3. Labelling of Phenolics in Developing
Fruits with [U-¹⁴C] Cinnamic acid**

3.3. Labelling of Phenolics in Developing Fruits with [U-¹⁴C] Cinnamic acid

It has been shown in 3.2 that the levels of glycosylated flavonoids and hydroxycinnamates were high in fruits of stage I. During the onset of capsaicin synthesis (stage II), these compounds decrease in concentration and may disappear completely. Also during stage II, apart from an active accumulation of capsaicin and lignin, glycosylated hydroxybenzoates and hydroxybenzaldehydes were also accumulated. The aim of this experiment was to investigate the flux of these intermediates in relation to the onset of capsaicin synthesis by following the incorporation of [U-¹⁴C] cinnamic acid into free, water soluble conjugated and saponifiable cell wall phenolics, and lignin.

In this and subsequent experiments, all the plants were grown in a controlled environment chamber as described in 2.1. This ensured a continuous supply of fruits and reduced the variation within the population. Fruits of plants grown under these conditions, begin to synthesize capsaicin at about d.15 and are orange red in colour by d.50. Fruits between d.0 and d.15 were assigned to stage I, those between d.15 and d.35 to stage II and after d.35 to stage III.

3.3.1. Distribution of [¹⁴C] in free phenolics, water soluble conjugated phenolics, saponifiable cell wall phenolics and lignin (Fig. 3.3.1)

In this experiment three fruits at d. 10, 15, 20, 25, 30, 35, and 40 were each fed for 6h with 0.76μCi [U-¹⁴C] cinnamic acid [SA=24 mCi.mmol⁻¹] in 20μl of 0.05M phosphate buffer at pH 7.0. After labelling the phenolics were extracted from each fruit and fractionated according to the method described in 2.3.2.1. Measurement of the radioactivity in each phenolic fraction was as set out in 2.3.6.2.iii. In fruits of d.10 (stage I) 90% of the total [¹⁴C] supplied was present in the water soluble conjugated phenolics. No significant amount of radioactivity was found in free phenolics. Only 5 and 8 % of the total [¹⁴C] supplied was present in lignin and the saponifiable cell wall phenolics respectively. In fruits of stages II and III, 15 -22% of

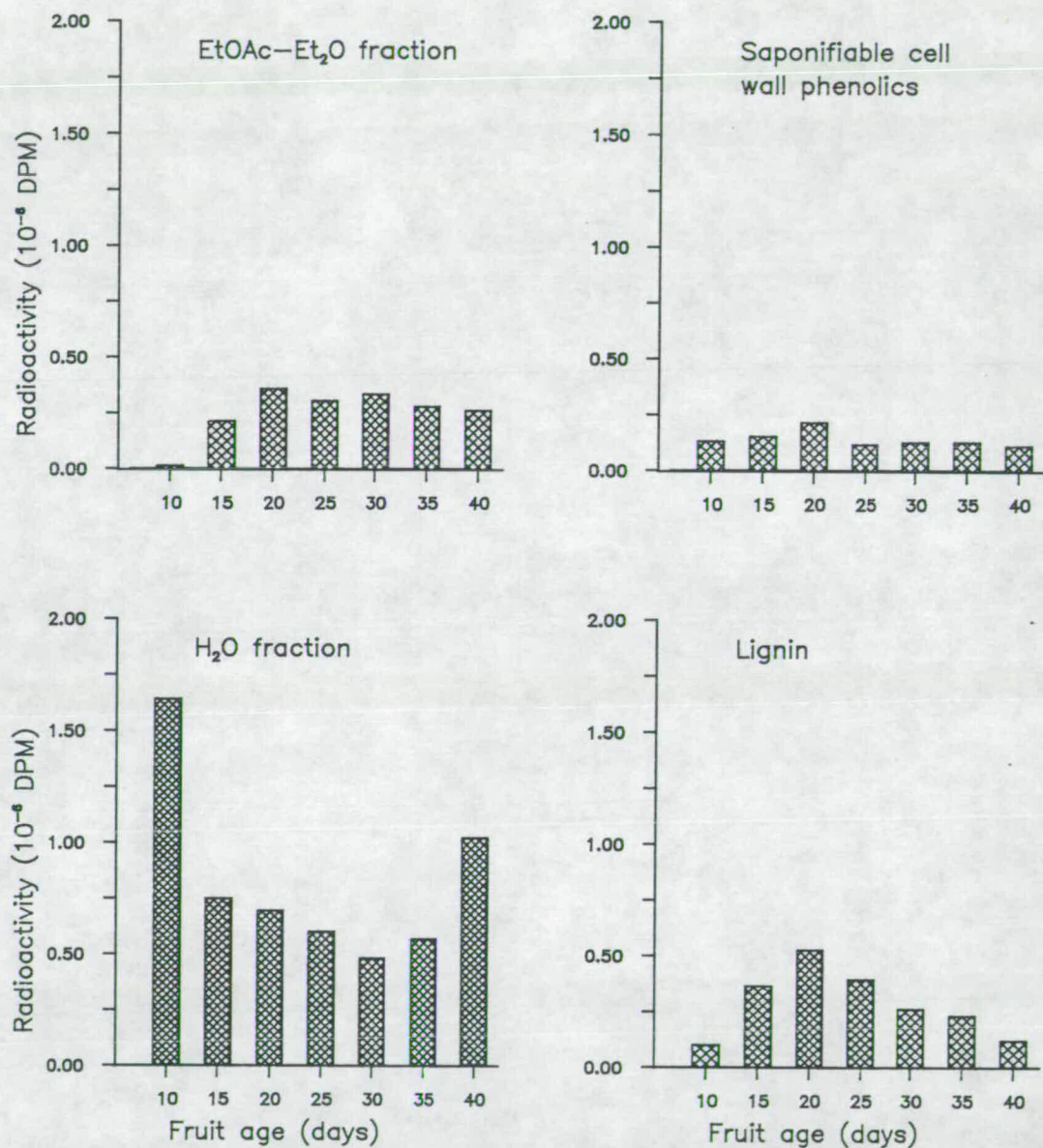


Fig. 3.3.1. Changes in the incorporation of $[U-^{14}C]$ cinnamic acid into free phenolics (EtOAc-Et₂O fraction), water soluble conjugated phenolics (H₂O fraction), saponifiable cell wall phenolics and lignin in developing fruits. Each fruit was fed with $0.76 \mu\text{Ci}$ $[U-^{14}C]$ cinnamic acid for 6h. Data are presented as an average of three replicates on a *per* fruit basis.

the radioactivity was in free phenolics. The level of incorporation of [U-¹⁴C] cinnamic acid into the water soluble conjugated phenolics in fruits of stage II was much lower than in stage I. Only 30-50% of the radioactivity was found in these phenolics. However, in fruits of stage-III (d.40) the level of [¹⁴C] in the water soluble conjugated phenolics was higher than in the fruits of stage II. The incorporation of [U-¹⁴C] cinnamic acid into the saponifiable cell wall phenolics was similar at all developmental stages, approximately 10% of the total [¹⁴C] supplied. The incorporation of [U-¹⁴C] cinnamic acid into lignin was at its highest level in the fruits of d.20 (32% of total radioactivity supplied), then decreased. By d.40 (stage III) there was still some incorporation of [U-¹⁴C] cinnamic acid into lignin (7%).

3.3.2. Incorporation of [U-¹⁴C] cinnamic acid into free phenolics (Fig.3.3.2)

Free phenolics were separated by one dimensional TLC-Silica gel using solvent system II and the radioactive spots detected using autoradiography. Seven free phenolics were detected, two of the phenolics were identified as capsaicin and cinnamic acid. The rest were not identified and were given numbers in sequence according to their R_f values, small numbers correspond to high R_f values. Free phenolic-1 is possibly a mixture of p-coumaric and ferulic acid which have close R_f values with the TLC solvent system used.

In 10d. fruits (stage I), all the [U-¹⁴C] cinnamic acid added was metabolised and only free phenolic-5 was detected at a very low activity. After the onset of capsaicin synthesis (c. d.15), [¹⁴C] was also detected in the other free phenolics. The incorporation of [U-¹⁴C] cinnamic acid into capsaicin in fruits of stage II increased sharply and reached a maximum level at d.30 then the level of incorporation decreased. By d.40 (stage III) there was still some incorporation of [U-¹⁴C] cinnamic acid into capsaicin. The level of radioactivity in free phenolics-1 and 2 was low in fruits early in stage II and higher in the later stages of II and in stage III. The pattern of incorporation of [U-¹⁴C] cinnamic acid into free phenolic-3 was similar to that of capsaicin, but the level of incorporation was much lower. There was significant activity of [¹⁴C] in free phenolic-4 in fruits of stage II and the activity was very low

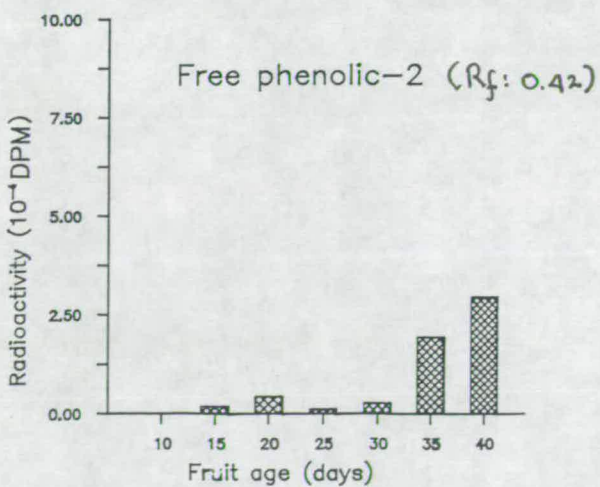
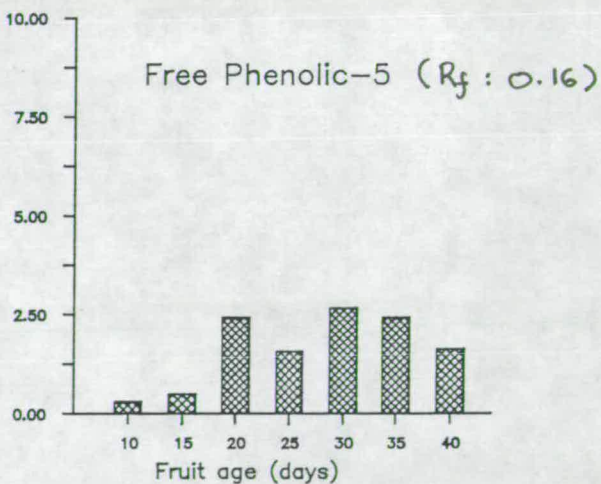
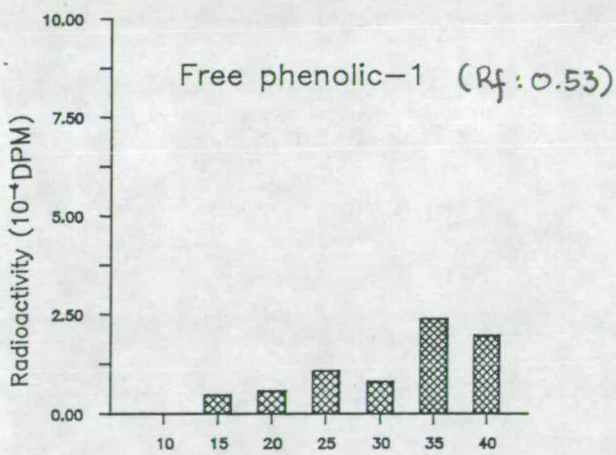
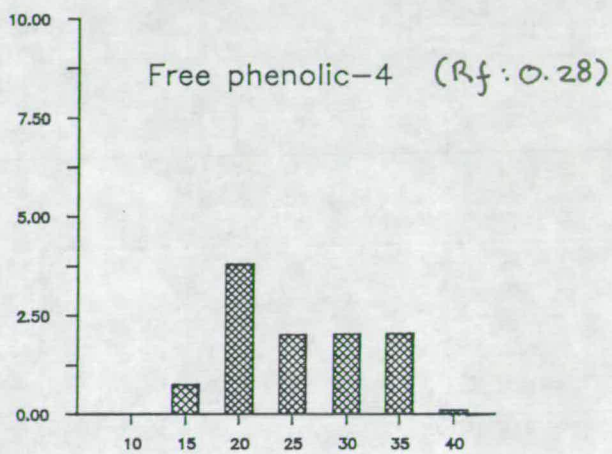
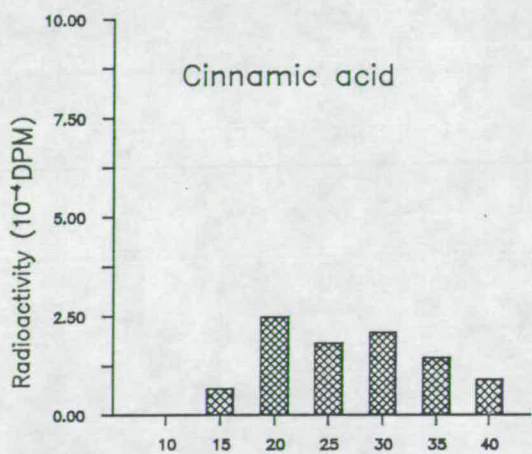
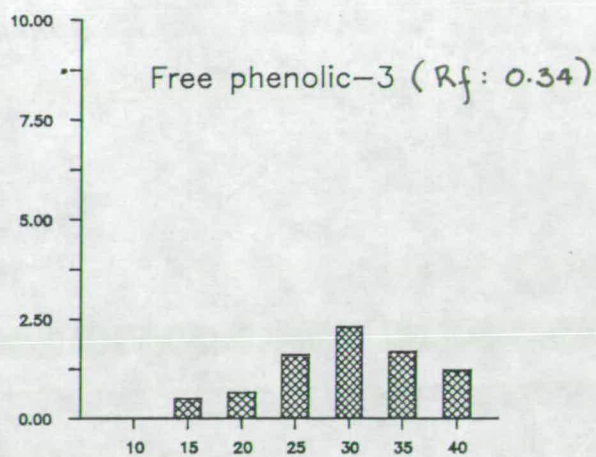
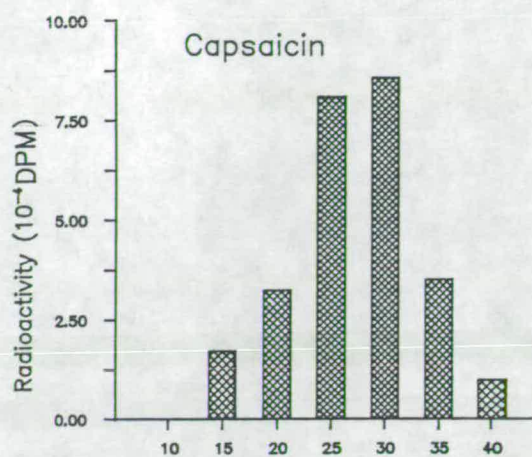


Fig. 3.3.2. Changes in the incorporation of [U-¹⁴C] cinnamic acid into capsaicin and other free phenolics in developing fruits. Each fruit was fed with 0.76 μ Ci [U-¹⁴C] cinnamic acid for 6 h. Data are presented as an average of three replicates on a per fruit basis

in fruits of stages I and III. A low activity of [^{14}C] was detected in free phenolic-5 at stage I and the activity was higher in fruits of stages II and III. In all developmental stages, the level of [$\text{U-}^{14}\text{C}$] cinnamic acid left after 6h. labelling was low.

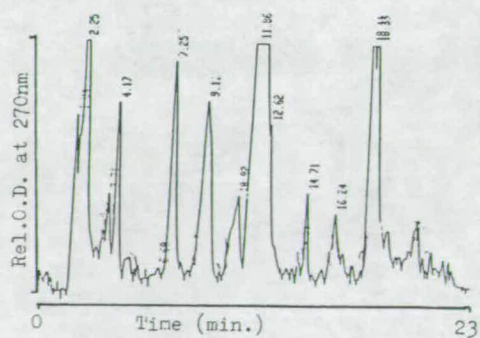
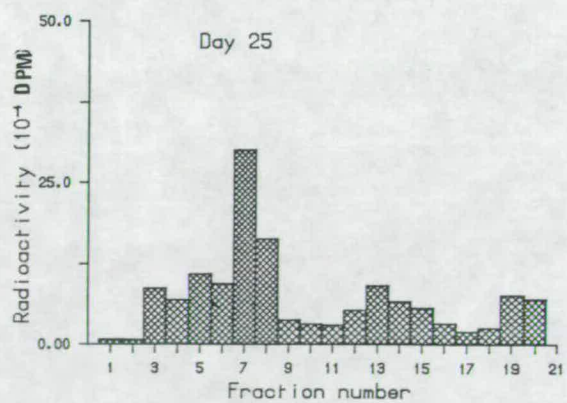
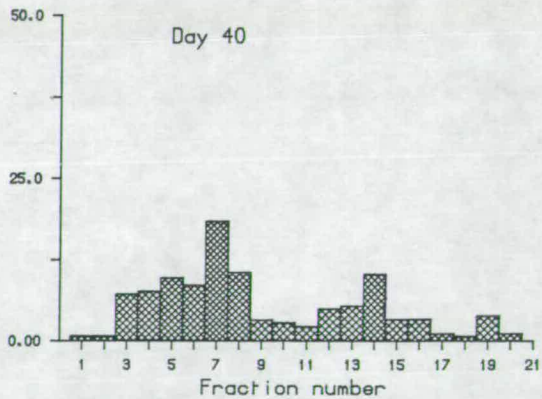
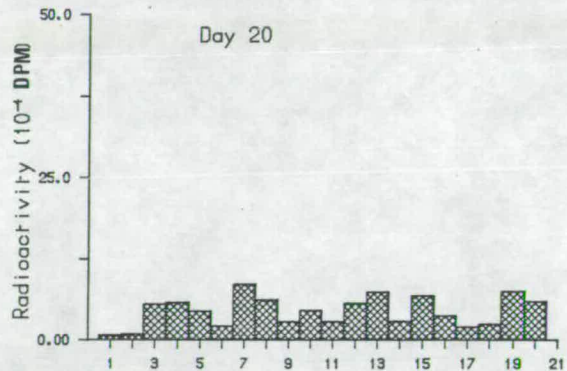
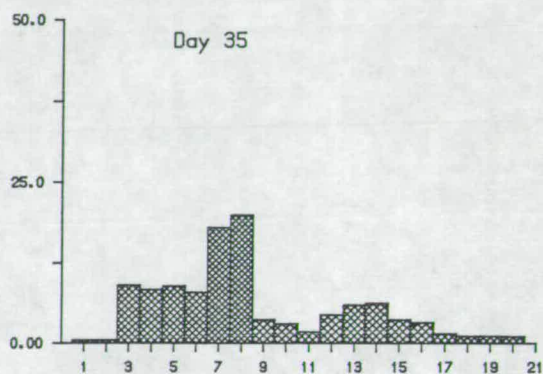
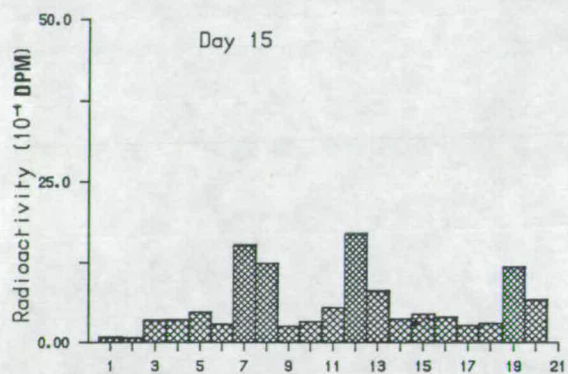
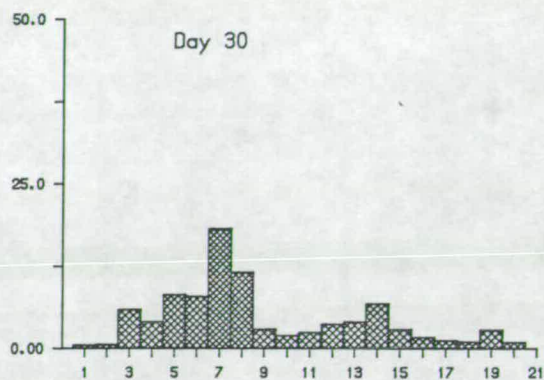
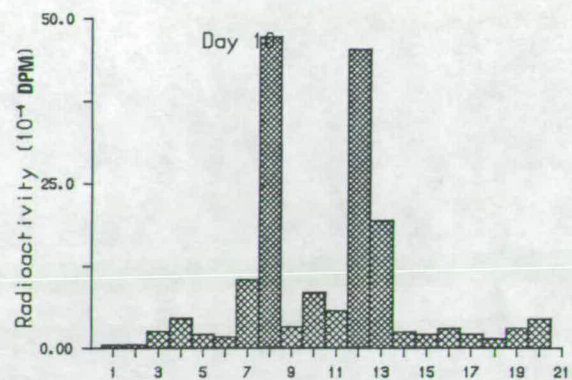
3.3.3. The incorporation of [$\text{U-}^{14}\text{C}$] cinnamic acid into the water soluble conjugated phenolics (Fig. 3.3.3)

The radioactivity of phenolics in the water fraction was measured following the separation of the compounds using an HPLC chromatograph and a fraction collector. In the fruits of stage I, 30 and 40% of the [^{14}C] supplied was found in caffeoyl and 3,4-dimethoxycinnamoyl glycoside respectively (see Fr.8 and Fr.12-13). This indicates that these compounds are being actively synthesized. Only a small part of the radioactivity was found in p-coumaroyl glycoside (see Fr.11). Flavonoids are not the major component of water soluble conjugated phenolics in fruits of plants grown under the conditions described. Indeed the small proportion of [^{14}C] found in flavonoids (see Fr.19 and 20) confirms the remoteness of this biosynthetic pathway from cinnamic acid.

By d.15, the incorporation of [$\text{U-}^{14}\text{C}$] cinnamic acid into caffeoyl and 3,4-dimethoxycinnamoyl glycoside was much lower than at d.10. However, there was a small increase in the level of incorporation into flavonoids. When the incorporation of [$\text{U-}^{14}\text{C}$] cinnamic acid into lignin was at its highest level (see d.20 in Fig. 3.3.1), the incorporation into the water soluble conjugated phenolics was low. By d.25, there was a shift in the incorporation of [$\text{U-}^{14}\text{C}$] cinnamic acid into the water soluble conjugated phenolics, Fr.7 which was undetected by HPLC, was highly labelled. Compounds with low R_t values also became labelled. Most of these compounds are in a group of $\text{C}_6\text{-C}_1$ phenolics such as glycosides of hydroxybenzoic acids, hydroxybenzaldehydes, and hydroxybenzylalcohols. The labelling pattern of the water soluble conjugated phenolics in fruits of d.30 to d.40 was similar to that of d.25 fruits.

Fig. 3.3.3.

Changes in the distribution of [^{14}C] in a series of fractions collected every min. from the HPLC of the water soluble conjugated phenolics in developing fruits. The peaks shown in the chromatogram are: unknown phenolic glycoside I [Rt 2.25], vanillic acid glycoside [Rt 4.17], caffeoyl glycoside [Rt 7.25], unknown phenolic glycoside II [Rt 9.11], p-coumaroyl glycoside [Rt 10.92], 3,4-dimethoxycinnamoyl glycoside [Rt 11.86], unknown phenolic glycoside III [Rt 14.71], flavonoids [Rt 16.24 to 18.33]. Data are presented as an average of three replicates on a *per* fruit basis.



3.3.4. Summary

Fruits of *Capsicum frutescens* actively metabolized cinnamic acid at all developmental stages. The final products, however, were different in the three developmental stages. During stage I, cinnamic acid was mainly incorporated into the hydroxycinnamoyl glycosides, while in fruits of stage II it was directed mainly into capsaicin, lignin and C₆-C₁ phenolic glycosides. In the last stage it was mainly incorporated into C₆-C₁ phenolic glycosides. Labelled free phenolics were detected in fruits of stages II and III, but not in fruits of stage I, these may act as intermediates of C₆-C₁ phenolic glycosides, capsaicin and lignin biosynthesis. as their presence after 6h labelling coincided with the high incorporation of [¹⁴C] into the products. This possibility was further tested in 3.4.

**Section 3.4. Labelling of Phenolics in Fruits at
The Onset of Capsaicin Synthesis [Stage II]**

3.4. Labelling of Phenolics in Fruits at The Onset of Capsaicin Synthesis [Stage II]

The evidence already available from previous investigations (Bennet and Kirby, 1968; Leete and Loudon, 1968, Hall *et al.*, 1987, Holden *et al.*, 1987a) and results presented in 3.3 suggest that free phenolics are the intermediates in capsaicin biosynthesis. However, there is a distinct possibility that water soluble conjugated phenolics may also be involved as intermediates in phenylpropanoid metabolism. Harborne and Corner (1961) have proposed that hydroxycinnamoyl glycosides may act as biosynthetic intermediates in plants. Indeed it has been shown by Moriguchi *et al.*, (1988) that cinnamoyl and p-coumaroyl glucose are precursors of chlorogenic acid (caffeoyl quinic acid). It has also been suggested that cell wall bound phenolics are released during fruit softening and may be used as precursors for the synthesis of capsaicin (Holden *et al.*, 1987a). In this present study, the possibility that free, water soluble conjugates and saponifiable cell wall phenolics may all act as precursors in phenylpropanoid metabolism, especially leading to capsaicin synthesis, has been investigated using the radioactive tracers [U-¹⁴C] cinnamic acid, [³H] and [¹⁴C] p-coumaric acid, and [¹⁴C] caffeic acid.

3.4.1. Time Course Labelling of Phenolics in 15d. old Fruits with [U-¹⁴C] Cinnamic acid

It has been shown in 15d. old fruits (3.3), that capsaicin, lignin, hydroxycinnamoyl glycosides and saponifiable cell wall phenolics were all labelled after a 6h pulse with 0.76μCi [U-¹⁴C] cinnamic acid (SA 24mCi.mmol⁻¹). In this experiment, an attempt was made, using fruits of this age, to study the relationship between the synthesis of the hydroxycinnamoyl glycosides, saponifiable cell wall phenolics, lignin and capsaicin.

Eighteen attached fruits were each fed with 0.76μCi [U-¹⁴C] cinnamic acid (SA 24mCi.mmol⁻¹) in 20μl of 0.05M phosphate buffer at pH 7.0. After 10, 20, 40, 60, 180 and 360min. from the addition of [U-¹⁴C] cinnamic acid, three fruits were

removed and the phenolics in each fruit extracted and fractionated according to the methods described in 2.3.2.1. The radioactivity in free, water soluble conjugates and saponifiable cell wall phenolics and in lignin were all measured using LSC (see 2.3.6.2.iii).

3.4.1.1. Distribution of [^{14}C] in free, water soluble conjugates and saponifiable cell wall phenolics and in lignin

The radioactivity in all phenolic fractions was measured in order to obtain a balance sheet of [^{14}C] in the fruits after the addition of [$\text{U-}^{14}\text{C}$] cinnamic acid. The results presented in Fig. 3.4.1 show the change in the distribution of radioactivity in the different phenolic fractions over the time course. The level of radioactivity in free phenolics was very high at 10min. due to the presence of [$\text{U-}^{14}\text{C}$] cinnamic acid which had not been metabolized. However, the level decreased rapidly within 60min., indicating that the [$\text{U-}^{14}\text{C}$] cinnamic acid was being metabolized. Between 60 and 180min., the level of [^{14}C] in this fraction decreased slowly, then remained constant.

A rapid decrease in the radioactivity in the free phenolic fraction was paralleled by a substantial incorporation of [$\text{U-}^{14}\text{C}$] cinnamic acid into the water soluble conjugated phenolics (10 to 40 min.). Subsequently the level of [^{14}C] in water soluble conjugated phenolics remained constant between 40 and 360 min. suggesting that a steady state condition had been attained. Interconversion between metabolites and further metabolism of compounds in this fraction presumably take place in fruits during this stage.

The incorporation of [$\text{U-}^{14}\text{C}$] cinnamic acid into the saponifiable cell wall phenolics was very low at 10min. Subsequently the radioactivity in this fraction increased slowly until the end of the experiment. The level of incorporation into the saponifiable cell wall phenolics was low, with approximately 12% of the total [^{14}C] supplied present in this fraction at 360min. The incorporation of [$\text{U-}^{14}\text{C}$] cinnamic acid into lignin was also barely detectable at 10min. which is consistent with the remoteness of lignin from cinnamic acid. Between 20 and 40min., the radioactivity in lignin was still very low, then the

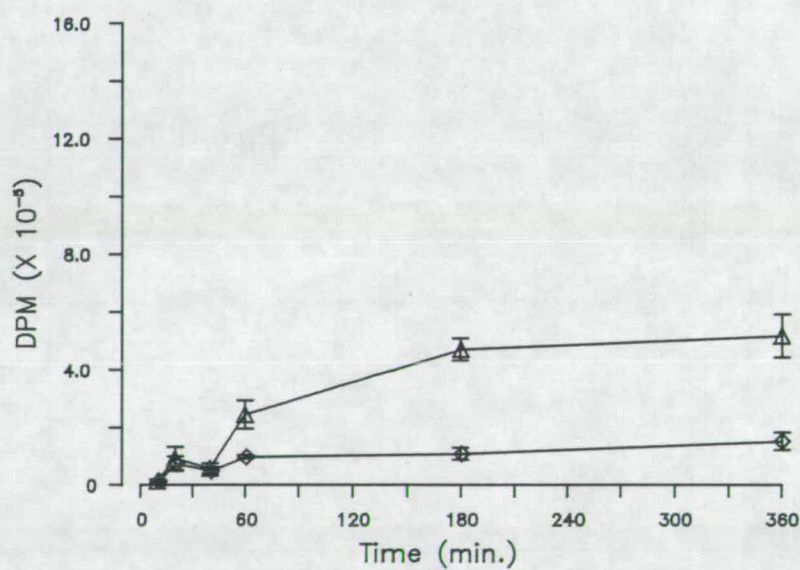
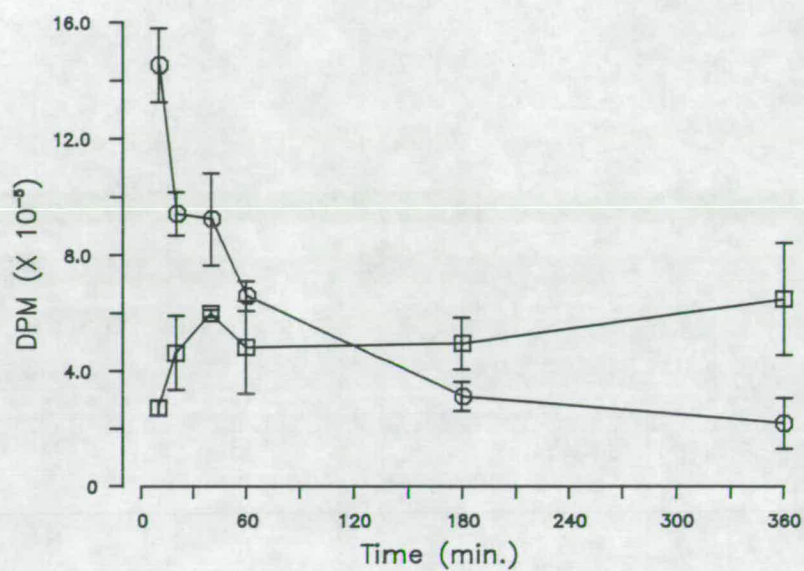


Fig. 3.4.1. Distribution of [^{14}C] in free phenolics [O], water soluble conjugated phenolics [□], sapnifiable cell wall phenolics [◇] and lignin [Δ] in 15d. old fruits fed with $0.76 \mu\text{Ci}$ [$\text{U}-^{14}\text{C}$] cinnamic acid. Data are presented on a *per* fruit basis with bars representing the SE of the mean of three replicates.

level increased rapidly until 180min. and remained constant until the end of the experiment. The fact that the level of radioactivity in the saponifiable cell wall phenolics and lignin reached a constant level suggests as expected that these compounds are not further metabolized. Indeed lignin is very likely to be the end product of metabolism (Schubert, 1973) and so far there has been no report suggesting the lignin is degraded in plants. The relatively low ^{increase} level of radioactivity in lignin and the saponifiable cell wall phenolics after 180min. is possibly due to the restricted availability of intermediates for synthesis. Indeed as already pointed out, at 180min. the radioactivity in free phenolic intermediates was very low (see Fig. 3.4.3).

3.4.1.2. Incorporation of [U-¹⁴C] cinnamic acid into free phenolics

Following fractionation of the MeOH extract into free phenolics (EtOAc-Et₂O fraction) and water soluble conjugated phenolics (aqueous fraction) the free phenolic extracts were separated by TLC and the radioactive spots visualized by autoradiography. For the purpose of identification, an extract of free phenolics from fruit labelled for 60min., was separated by a 2D-TLC. The 2D-TLC autoradiograph of this extract is shown in Fig. 3.4.2. Cinnamic, p-coumaric, caffeic and ferulic acid, and capsaicin were all present. In addition, four unknown free phenolics, i.e. free phenolics-A, B, C and D were also detected. Vanillin and vanillylamine, two of the proposed phenolic intermediates in capsaicin biosynthesis were not detected. The absence of radioactivity in vanillin is unexpected, however vanillylamine is polar and not present in the non-aqueous fraction. In an attempt to ascertain the role of labelled free phenolics synthesized from [U-¹⁴C] cinnamic acid in phenylpropanoid metabolism, the radioactivity in the individual phenolics was followed over the time course. The radioactivity of these phenolics was measured after separating them by TLC in solvent system II. The radioactivity of ferulic acid which coelutes with p-coumaric acid in this solvent system, was counted after separation of the ferulic acid from the other components of the extract by TLC using solvent system I. The radioactivity in each phenolic was counted using LSC (2.3.6.2.iii).

Fig. 3.4.3 shows the change in radioactivity of the free phenolics over the time

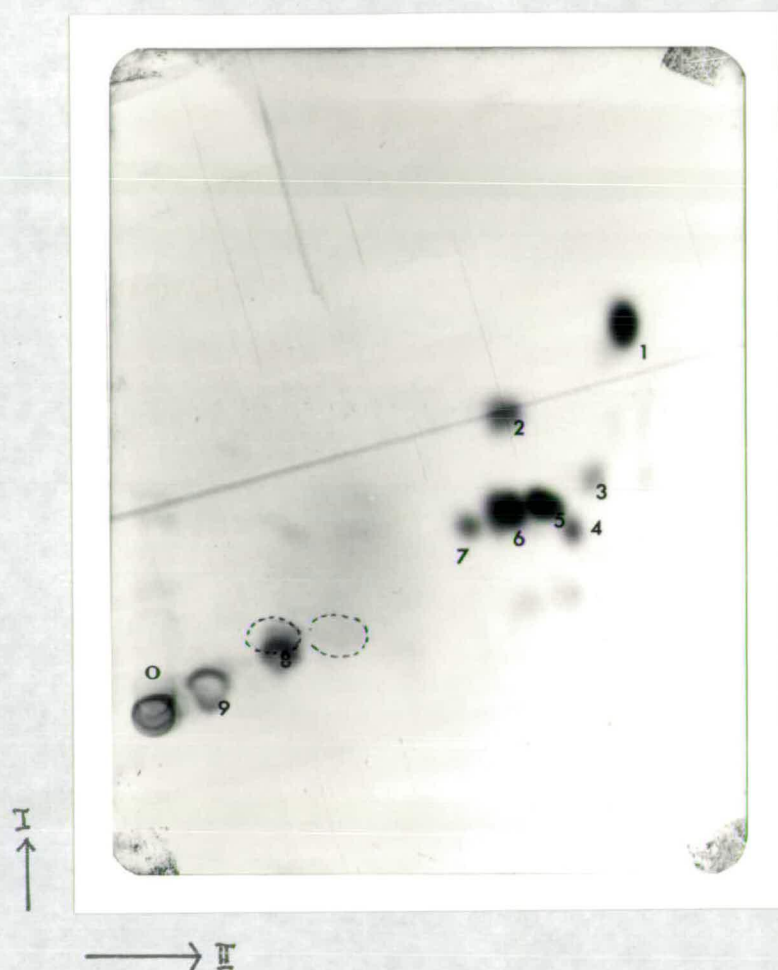


Fig. 3.4.2. 2D-TLC autoradiograph of free phenolics in 15 d. old fruits labelled with $0.76\mu\text{Ci}$ $[\text{U-}^{14}\text{C}]$ cinnamic acid for 60min. TLC was on Silica gel using solvent system I and II for the first and second dimensions respectively. Thirty microlitres of a 1.0 ml extract was applied together with a mixture of authentic markers to identify the radioactive spots. 1. Cinnamic acid, 2. ferulic acid, 3. capsaicin, 4. free phenolic-A, 5. free phenolic-B, 6. p-coumaric acid, 7. unknown phenolic, 8&9 free phenolics-C&D. Dashed spots are traces of *cis* and *trans* caffeic acid.

O : origin

course. [U-¹⁴C] cinnamic acid was rapidly taken up from the locus and within 180min., almost all of the tracer supplied had been metabolized. p-Coumaric and ferulic acid were both labelled rapidly and the [¹⁴C] in these compounds reached a maximal level between 40 and 60 min. then the levels decreased rapidly. The same pattern of labelling was observed with free phenolics-A, B, C and D. Caffeic acid, which only appeared as a faint spot on 2D-TLC autoradiograph (see Fig. 3.4.2), was not properly separated from free phenolic-C in either of the solvent systems. Therefore, the radioactivity in free phenolic-C was partly due to caffeic acid.

Capsaicin was also rapidly labelled leading to a significant level of [¹⁴C] in that compound within 10min., subsequently the level of radioactivity increased until 180min. and then remained constant. Labelling for a further 18h. (data not presented) did not lead to a decrease in the level of radioactivity in capsaicin. This was in agreement with the results of Hall *et al.* (1986) who showed that capsaicin in fruits of *Capsicum frutescens* was not further metabolized. It is interesting to note that the increase in incorporation of [U-¹⁴C] cinnamic acid into capsaicin^{after 60 min.} was paralleled by the disappearance of radioactivity in the other free phenolics.

The rapid incorporation of a radioactive precursor into a metabolite followed by its rapid disappearance provides good evidence that the metabolite is an active intermediate. It would also seem from the pattern of labelling observed, that apart from capsaicin, the free phenolics detected are active intermediates in phenylpropanoid metabolism. Indeed p-coumaric, caffeic and ferulic acid are well established as general intermediates in phenylpropanoid metabolism (Hanson and Havir, 1979; Barz *et al.*, 1985). Free phenolics-A, B, C and D are possibly intermediates involved in the biosynthesis of lignin, saponifiable cell wall phenolics and certain water soluble conjugated phenolics. But the possible involvement some of these unknown free phenolics in capsaicin biosynthesis cannot be excluded.

3.4.1.3. Incorporation of [U-¹⁴C] cinnamic acid into water soluble conjugated phenolics

The incorporation of [U-¹⁴C] cinnamic acid into water soluble conjugated

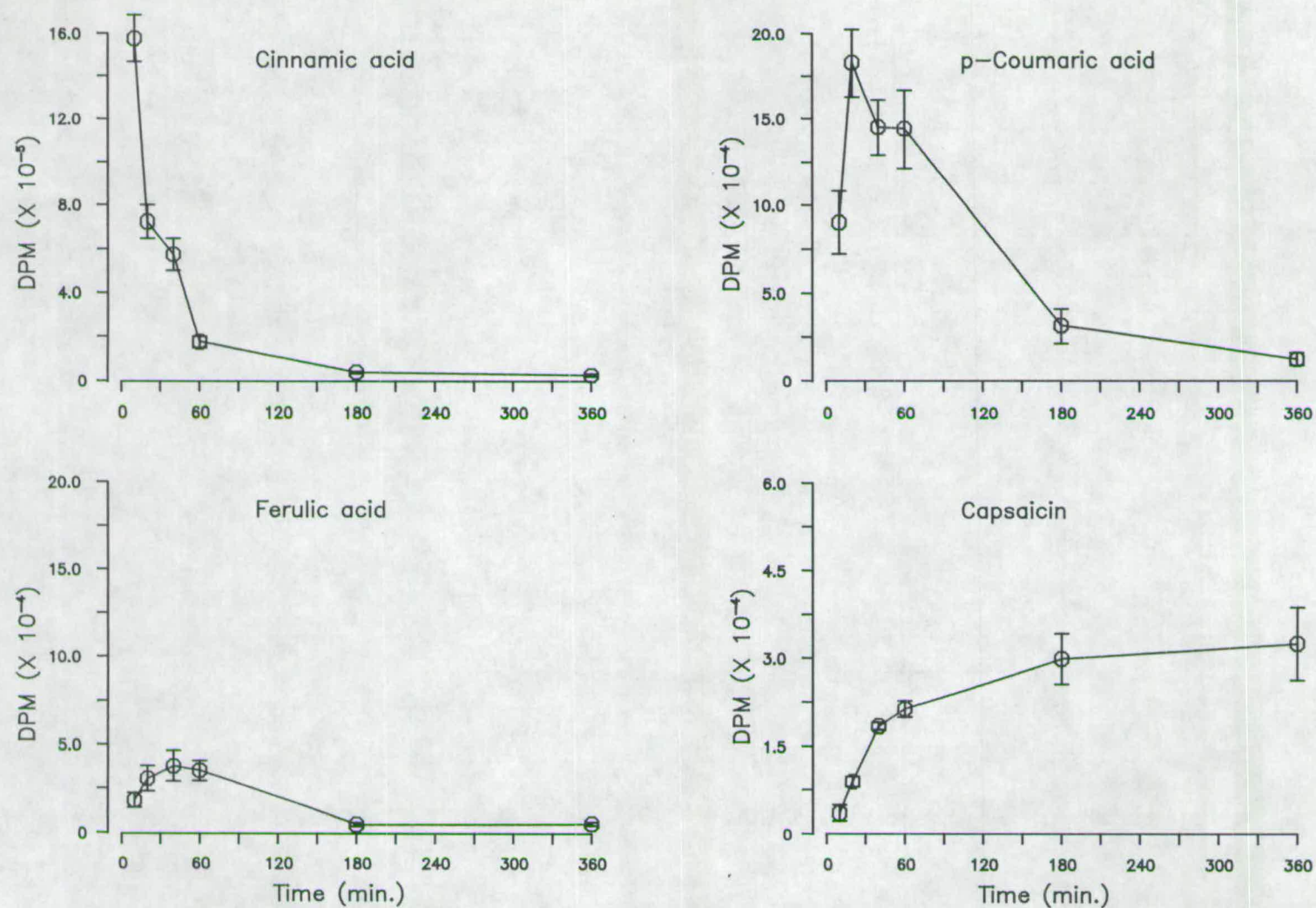


Fig. 3.4.3. Radioactivity of free phenolics in 15d. old fruits fed with 0.76 μ Ci [U-¹⁴C] Cinnamic acid. Data are presented on a *per* fruit basis with bars representing the SE of the mean of three replicates.

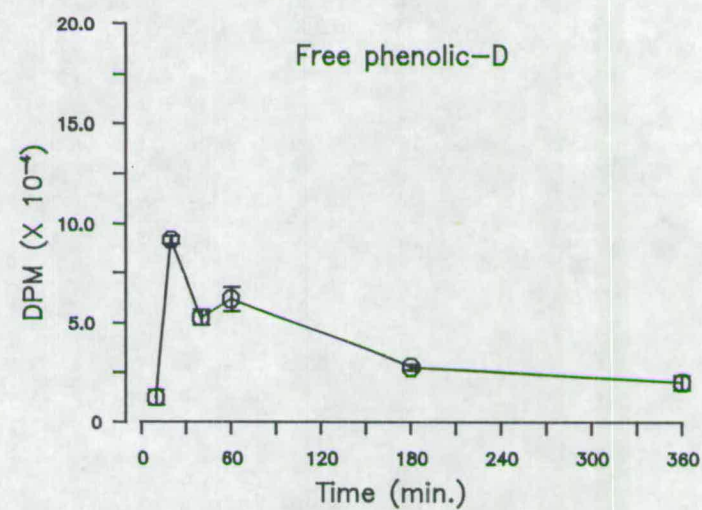
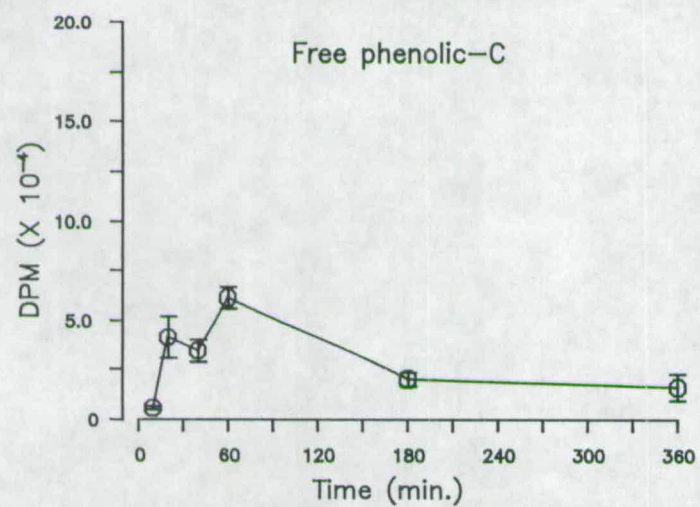
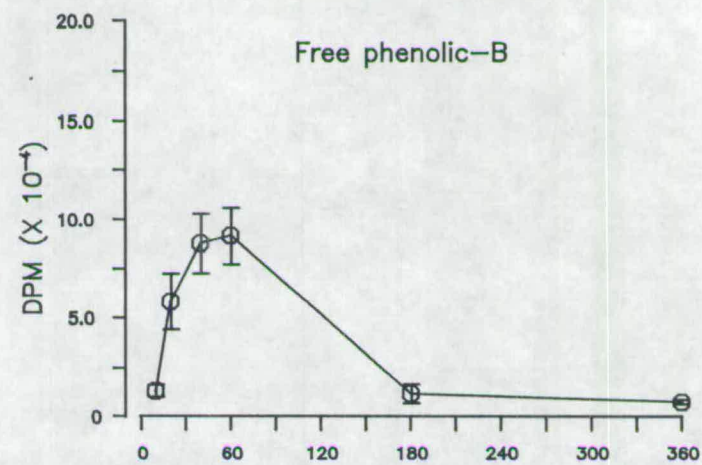
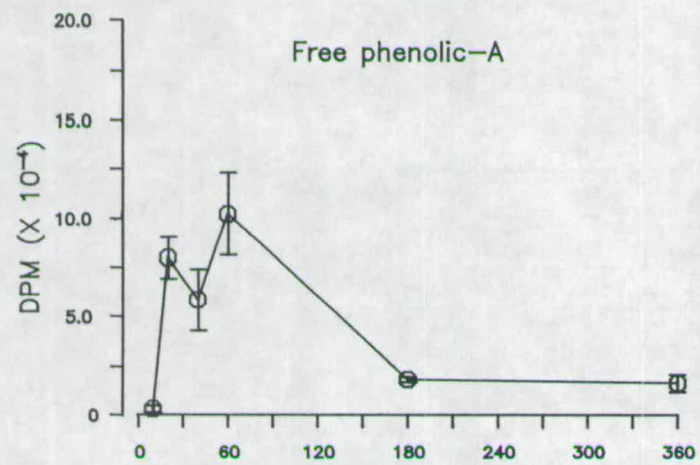


Fig. 3.4.3. (Continued)

phenolics was followed over a period of six hours. The incorporation of [U-¹⁴C] cinnamic acid into these compounds was determined after separation by HPLC. Fig. 3.4.4. shows the labelling pattern of six water soluble conjugated phenolics over the experimental period. p-Coumaroyl, caffeoyl and 3,4-dimethoxycinnamoyl glycosides were all rapidly labelled. The level of [¹⁴C] in these compounds increased until 60min., then remained constant. The radioactivity in the phenolic of peak-5 (described in Fig. 3.2.2, corresponds to Fr-3 in Fig. 3.4.4) and vanillic acid glycoside was first observed at 20min., then the levels increased until 180min. and finally remained constant. Radioactivity appeared in one unknown phenolic glycoside (peak of Rt 9.11 in Fig.3.3.3, corresponds to Fr-10 in Fig. 3.4.4) after 10min., and remained low until 40min., then increased between 40 and 60min., before settling down to a constant level. It is possible that the phenolic moiety of this compound is a member of the C₆-C₁ phenolics. Indeed, the UV spectrum of this compound is similar to phenolic glycoside-7 previously reported in cell cultures (see Fig. 3.1.12) which was thought to be a vanillin related compound. The incorporation of [U-¹⁴C] cinnamic acid into flavonoids was very low.

The pattern of incorporation of [U-¹⁴C] cinnamic acid into each of the water soluble conjugated phenolic was similar. Based on the evidence from the chemical structure, accumulation pattern and location of hydroxybenzoic acid glycosides and flavonoids accumulated in developing fruits (see 3.2) it is unlikely that these compounds are intermediates in capsaicin biosynthesis. The hydroxycinnamoyl glycosides, however, may act as precursors in capsaicin biosynthesis and this possibility was tested in the experiments reported in 3.6.

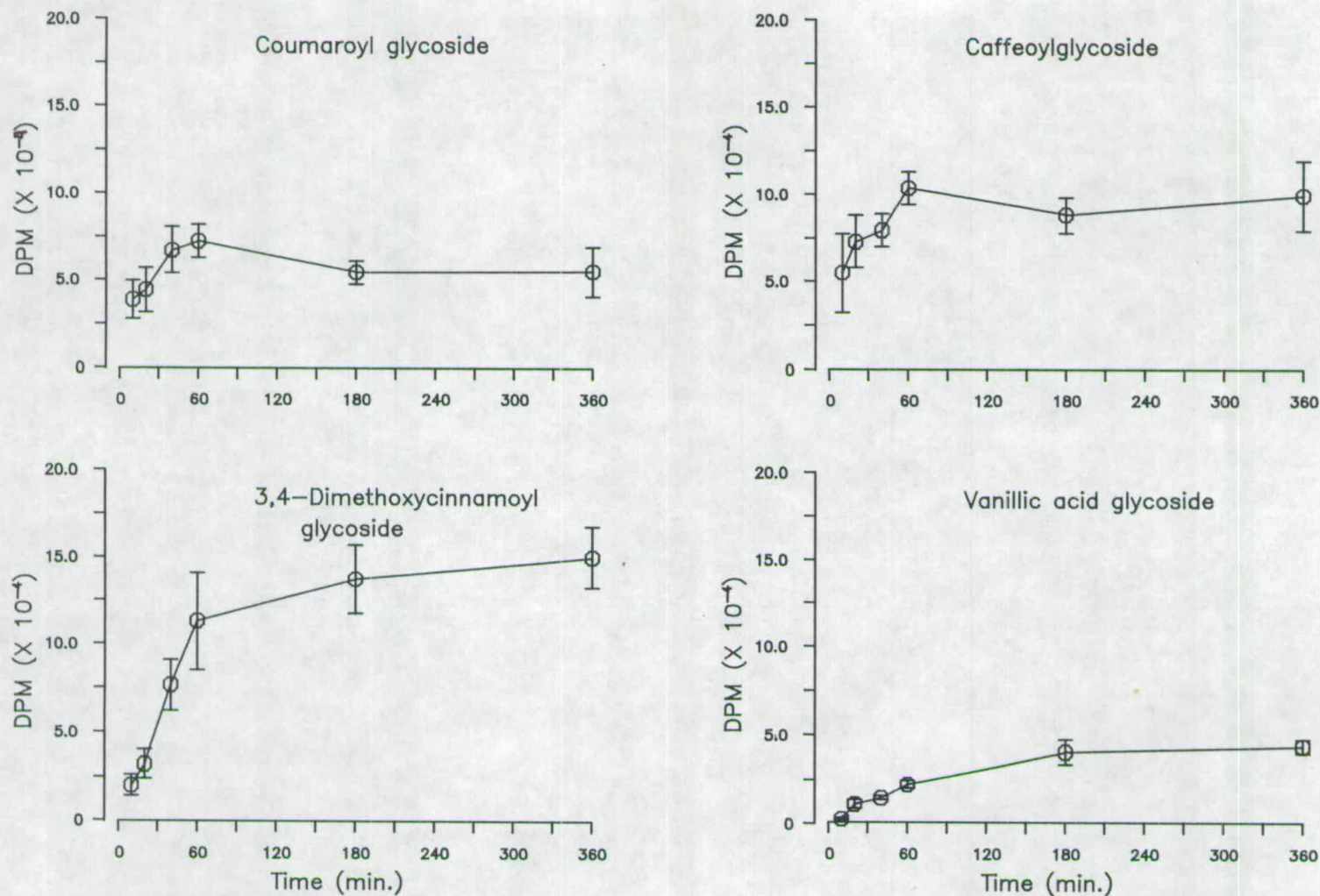


Fig. 3.4.4. Radioactivity of water soluble conjugated phenolics in 15d. old fruits fed with $0.76 \mu\text{Ci}$ [$U-^{14}\text{C}$] cinnamic acid. Fr-3 and Fr-10 correspond to compounds eluted between 2-3 and 9-10 respectively. Data are presented on a *per* fruit basis with bars representing the SE of the mean of three replicates.

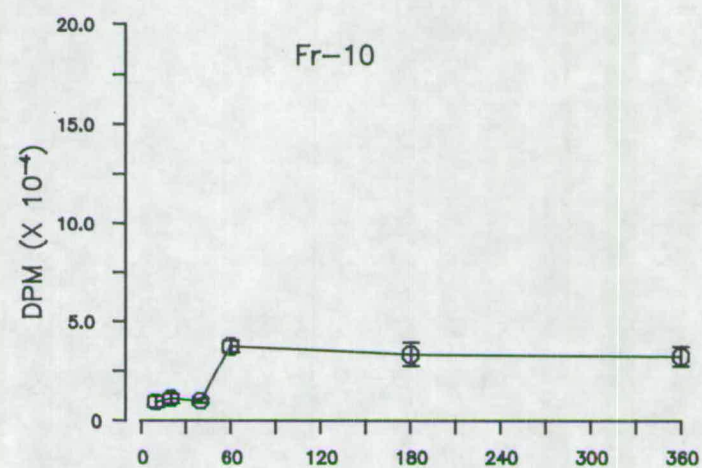
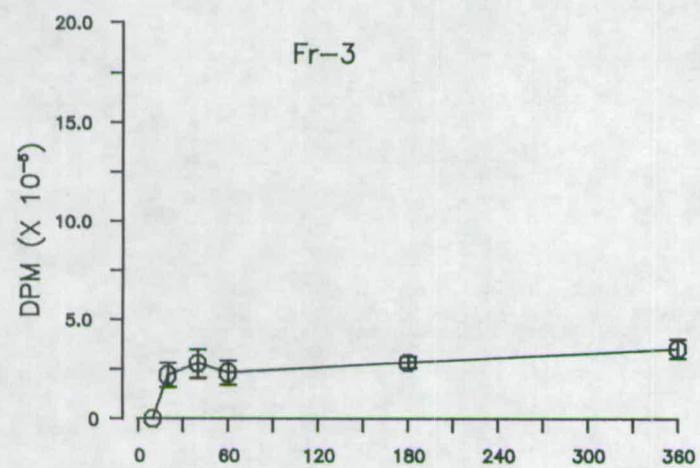


Fig.3.4.4. (Continued)

3.4.2. Labelling of Phenolics in 20d. old Fruits with [2-side chain- ^{14}C] p-Coumaric and Caffeic acid

The conversion of cinnamic acid to phenylalanine has been reported by Hall *et al.* (1987). In the experiment reported here, an attempt was made to discover whether any reverse reactions occurred at the other steps in the pathway, by feeding radioactive precursors further down the pathway than cinnamic acid. It was well understood that [^{14}C] of [2-side chain- ^{14}C] p-coumaric and caffeic acid will not be incorporated into capsaicin. These precursors, however, can be useful in studying the metabolic pathway between p-coumaric and ferulic acid and the pathway leading to the hydroxycinnamoyl glycosides, saponifiable cell wall phenolics and lignin. Particular attention in these experiments was paid to free phenolic intermediates, therefore fruits of d.20 which incorporate higher amounts of [U- ^{14}C] cinnamic acid into free phenolics than d.15 fruits were used.

3.4.2.1. One hour labelling with [2-side chain- ^{14}C] p-coumaric acid

In this experiment, two attached fruits were each fed with $1.4\mu\text{Ci}$ [2-side chain- ^{14}C] p-coumaric acid (SA 2.5mCi.mmol^{-1}) in $30\mu\text{l}$ of 0.05M phosphate buffer at pH 7.0. After 60min., the fruits were removed and the phenolics extracted and fractionated according to the methods described in 2.3.2.1. Free phenolics were separated by TLC using solvent system I and the chromatogram scanned with RITA (see Fig. 3.4.5). A MeOH solution containing a mixture of cinnamic, p-coumaric, caffeic and ferulic acid (1.0mg.ml^{-1} each) was also loaded to facilitate identification. It was expected, caffeic acid would be highly labelled within 1 h. of the addition of the tracer, but this was not the case. Only a very small amount of radioactivity was present in caffeic acid and the level of radioactivity in cinnamic acid was vanishingly small. Most p-coumaric acid added was converted into an unknown compound.

An attempt to identify this unknown compound was made by isolating the substance with TLC and the corresponding spot removed from the plate and developed with MeOH. The isolate was analysed using the electrophoretic method

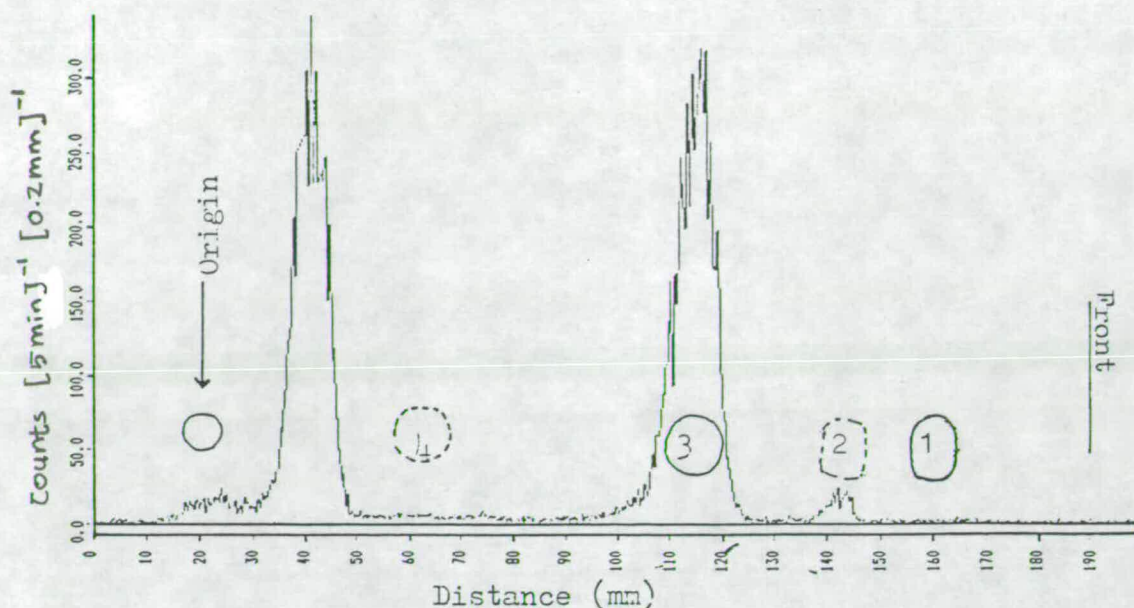


Fig. 3.4.5. TLC-RITA scan of the free phenolic extract of 20d. old fruit labelled with $1.4\mu\text{Ci}$ [2-side chain- ^{14}C] p-coumaric acid for 1h. TLC was on Silica using solvent system I. Twenty microlitres of a 1.0ml extract was applied together with a mixture of authentic markers. 1. Cinnamic acid, 2. ferulic acid, 3. p-coumaric acid and 4. caffeic acid.

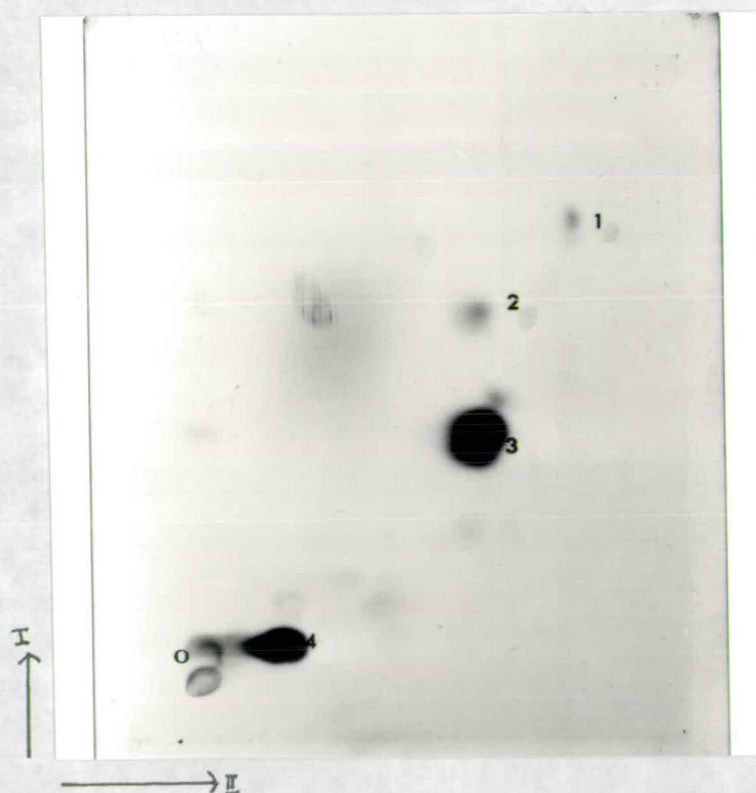


Fig. 3.4.6. 2D-TLC autoradiograph of the free phenolics in an extract of 20d. old fruit labelled with $1.4\mu\text{Ci}$ [2-side chain- ^{14}C] p-coumaric acid for 1h. TLC was on Silica gel using solvent system I and II for the first and second dimensions respectively. Twenty microlitres of a 1.0ml extract was applied together with a mixture of authentic markers. 1. Possibly 3,4-dimethoxy cinnamic acid, 2. ferulic acid, 3. p-coumaric acid and 4. free phenolic-E. o: origin

described by Fry (1988) to determine its behaviour in an electric field. Using a buffer at pH 5.5, this compound moved towards the anode while non acidic phenolics such as vanillyl alcohol and p-coumaroyl glucoside moved slightly towards cathode. This indicates that the compound still retains a carboxylic group. This compound (phenolic-E) was further identified by 2D-TLC followed by autoradiography (Fig. 3.4.6). Phenolic-E was shown not to be as chlorogenic or p-coumaroyl quinic acid,^{as} these two substances are retained at the origin with the TLC system used.

Although p-coumaric acid at a dose of 0.56 μ mol is apparently not toxic, this compound may be a detoxification product of p-coumaric acid. The fact that the level of radioactivity in ferulic acid (an intermediate two steps further down the pathway from p-coumaric acid) in this experiment with 15d. old fruits was much lower than in previous experiment using a lower activity of cinnamic acid (0.76 μ Ci) supports this possibility. A longer period of labelling with [2-side chain-¹⁴C] p-coumaric acid was also carried out to investigate the final fate of this compound (see 3.4.2.3).

3.4.2.2. One hour labelling with [2-side chain-¹⁴C] caffeic acid

A similar experiment to that described in 3.4.2.1 was carried out with [2-side chain-¹⁴C] caffeic acid. Since only a small amount of [2-side chain-¹⁴C] caffeic acid was obtained from the synthesis described in 2.5.4.3, only 0.42 μ Ci of the precursor in 50 μ l of 0.05M phosphate buffer at pH 7.0 was fed to each fruit. Analysis was as described in 3.4.2.1.

One hour after feeding, browning on the surface of the seeds was observed. Unlike the experiment with [2-side chain-¹⁴C] p-coumaric acid in which p-coumaric acid still retained 32% of the total radioactivity supplied, after one hour, in this experiment, only 7% of [¹⁴C] remained in caffeic acid. The radioactivity in the other compounds was much lower than in caffeic acid (see Fig. 3.4.7). Most of the caffeic acid fed has possibly been detoxified by the cells to produce MeOH insoluble materials. As for [2-side chain-¹⁴C] p-coumaric acid, [2-side chain-¹⁴C] caffeic acid

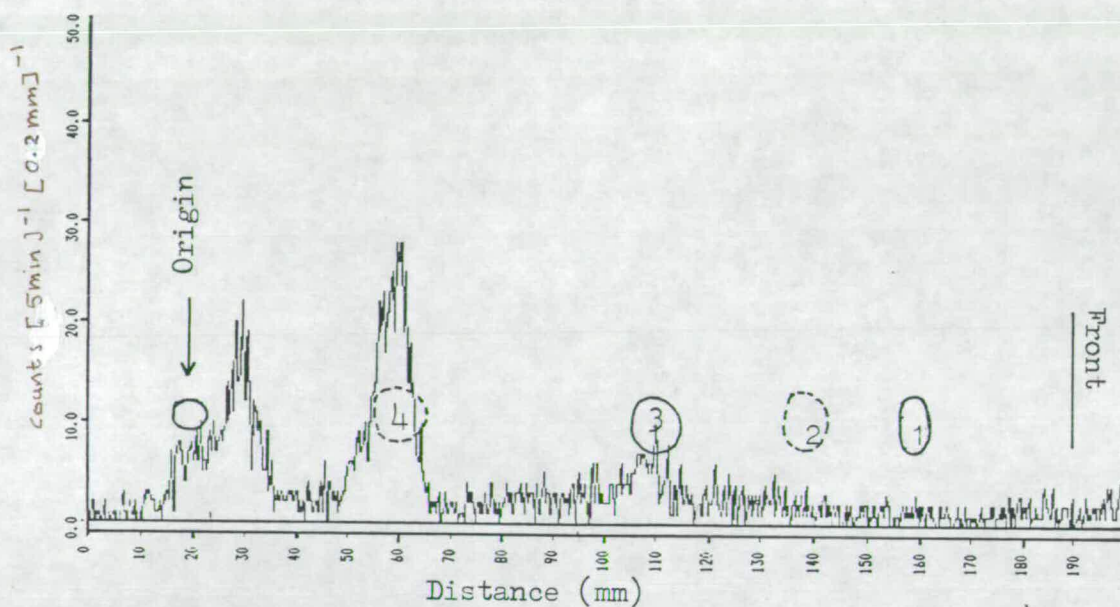


Fig. 3.4.7. TLC-RITA scan of the free phenolics in an extract of 20d. old fruit labelled with $0.42\mu\text{Ci}$ [2-side chain- ^{14}C] caffeic acid for 1h. TLC was on Silica gel using solvent system I. Forty microlitres of a 1.0ml extract was applied together with a mixture of authentic markers. 1. Cinnamic acid, 2. ferulic acid, 3. p-coumaric acid and 4. caffeic acid.

was also converted into an unknown compound (phenolic-F). No radioactivity was found in ferulic or cinnamic acid. There was, however, a low amount of radioactivity (4471 DPM = 0.5% of total supplied) in the spot which coelutes with p-coumaric acid. The identity of this compound was not determined and labelling with a higher SA of caffeic acid is necessary to confirm the back reaction from caffeic acid into p-coumaric acid.

3.4.2.3. Twenty four hour labelling with [2-side chain-¹⁴C] p-coumaric acid

To investigate the final fate of the phenolic-E which was rapidly produced from [2-side chain-¹⁴C] p-coumaric acid, a longer period of labelling was carried out and the results compared with those from the 1h. labelling experiment. The extraction procedure was as described in 3.4.2.1, but without further fractionation into free and water soluble conjugated phenolics. Following evaporation of the extract, the residue was taken up in 2.0ml MeOH, subsequently a 20µl sample was applied on TLC-Silica gel, developed with solvent system-I and the chromatogram scanned with RITA. In this TLC system, water soluble conjugated phenolics are retained at the origin. The radioactivities of separated components were counted using LSC (see 2.3.6.2.iii). The radioactivity of the saponifiable cell wall phenolics and lignin was also measured.

Table 3.4.1. shows the differences in the distribution of [¹⁴C] between 1h. and 24h. labelling. The radioactivity of phenolic-E after 24h. labelling was still very high but lower than with the 1h. labelling. This indicates that phenolic-E is further metabolized slowly. The extremely high count in saponifiable cell wall phenolics (20% of total [¹⁴C] supplied), may explain the final fate of phenolic-E. The higher count in the water soluble conjugated phenolics after 24h. is possibly due to the incorporation of p-coumaric, caffeic and ferulic acid. However, possible conversion of phenolic-E into a water soluble conjugate cannot be excluded.

Table 3.4.1. Comparison of the distribution of [^{14}C] in phenolics of 20d. old fruits following 1h. and 24h. labelling with 1.4 μCi [2-side chain- ^{14}C] p-coumaric acid.

Compounds	1h. (DPM)	24h.(DPM)
p-Coumaric acid	504,000	18,000
Phenolic-E	903,000	669,000
Water soluble conjugated phenolics	199,000	512,000
Saponifiable cell wall phenolics	81,000	527,000
Lignin	112,000	152,500
Total	1,799,000	1,868,500

Note: Data are presented as an average of two replicates on a *per* fruit basis

Although fruit at this stage are actively synthesizing lignin (3.3.1), the radioactivity in lignin after 24h. was only slightly higher than after 1h. It is well established (Grisebach, 1981) that lignin precursors are synthesized in the cytoplasm. Therefore, the low incorporation of the [2-side chain- ^{14}C] p-coumaric acid into lignin in 24h. labelling possibly suggests that the tracer added was mainly converted into phenolic-E, subsequently into saponifiable cell wall phenolics and these reactions take place in the cell walls.

3.4.3. Time Course Labelling of Phenolics in 20d. old Fruits with a high SA radioactive p-Coumaric acid

It has been suggested in 3.4.1 that free phenolics are more likely ^{than glycosylated phenolics} to be the direct intermediates in capsaicin biosynthesis. An attempt to study the metabolic pathway between p-coumaric and ferulic acid has been made in 3.4.2. p-Coumarate added into the fruits was rapidly converted into phenolic-E and only a very small part into caffeic acid. In these experiments, the possible role of phenolic-E in capsaicin biosynthesis was investigated. An attempt to detect all proposed intermediates between p-coumaric acid _{and capsaicin} was also made by following the metabolism of ring labelled

radioactive p-coumaric acid added into 20d. old fruits for 24h.

3.4.3.1. Labelling with [3,5-³H] p-coumaric acid

[3,5-³H]-p-Coumaric acid synthesized according to the method described in 2.5.4. has a SA of 51Ci.mmol⁻¹. By using this precursor, the possible toxic effect of p-coumaric acid can be avoided. In this experiment, 12 attached fruits were each fed with 1.7X10⁶CPM [3,5-³H] p-coumaric acid in a 40μl solution of 0.05M phosphate buffer at pH 7.0. After 1, 5, 60 and 360min., three fruits were harvested and the phenolics in each fruit extracted and fractionated according to methods described in 2.3.2.1. Free phenolics were separated by TLC using solvent system I and the radioactive chromatogram scanned with RITA. A ten microlitre solution containing a mixture of cold capsaicin intermediates (1.0mg.ml⁻¹ in MeOH) was also loaded onto the origin with the extract to facilitate identification of radioactive spots. Radioactivity of the separated phenolics was counted using LSC as described in 2.3.6.2.iii. The water soluble conjugated phenolics were separated by HPLC according to the method described in 2.3.4 and the separated phenolics collected and their radioactivity counted using LSC.

i. Radioactivity in free phenolics

Fig. 3.4.8 shows the change in radioactivity of free phenolics over the time course. There was no significant decrease in the radioactivity of p-coumaric acid within 1h. and there was still a significant level of radioactivity in this precursor after 6h. A significant level of radioactivity was detected in caffeic acid which reached a maximum level after 5min. A very low level of radioactivity was found in ferulic acid. After 6h. the radioactivity in capsaicin was barely above the level of the blank. Even after feeding with 3.4X10⁶CPM tracer for 24h., only 3,600CPM (0.01%) [³H] was present in capsaicin. Phenolic-E was not detected following this labelling. This result could support the idea that phenolic-E is a detoxification product of p-coumaric acid. However, there is another possibility: the tritium at positions 3 and 5 are removed from the precursor during the formation of phenolic-E from p-coumaric acid.

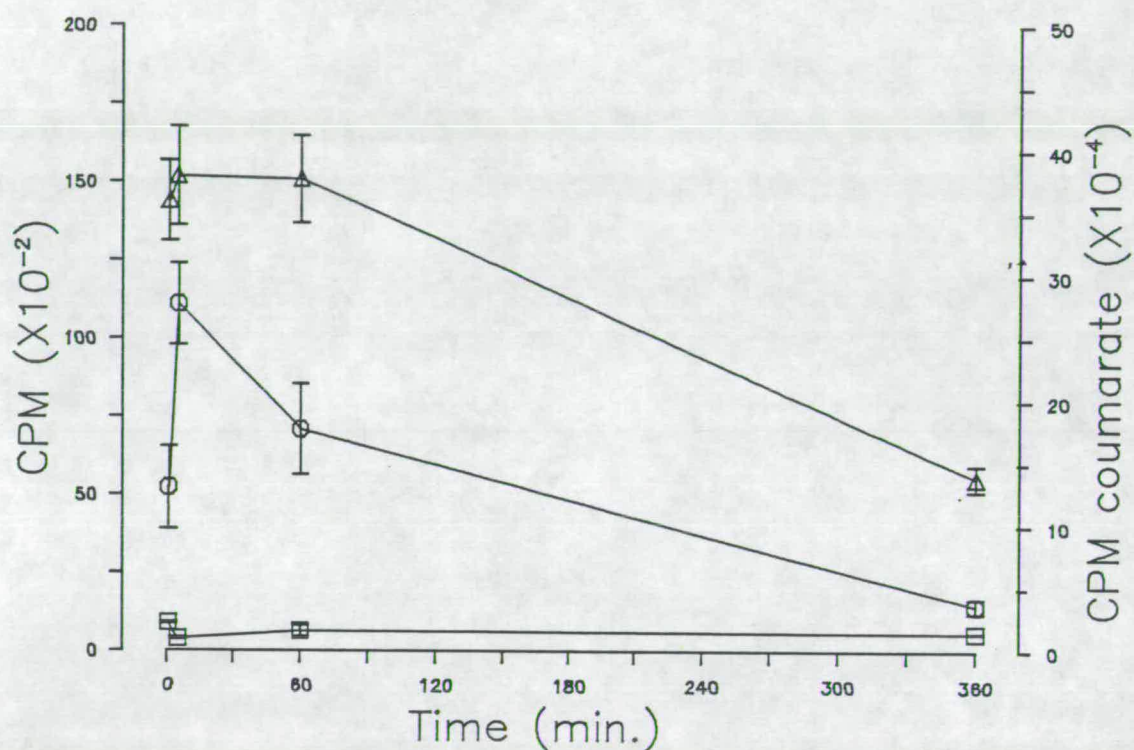


Fig. 3.4.8. Radioactivity of p-coumaric acid [Δ], caffeic acid [O] and ferulic acid [□] in 20d. old fruits fed with 1.7×10^6 CPM [3,5- ^3H] p-coumaric acid. Data are presented on *per* fruit basis with bars representing the SE of the mean of three replicates.

The presence of a high count in p-coumaric acid after a 6h. labelling period with 2.5 μ Ci [3,5- 3 H] p-coumaric acid, may be due to the low uptake of p-coumaric acid into the cells at pH 7.0 and would support the suggestion presented in 3.4.2 that 1.4 μ Ci [2-side chain- 14 C] p-coumaric acid (SA 2.5mCi.mmol $^{-1}$) added into fruits is metabolized mainly in the cell walls.

ii. Radioactivity in the water soluble conjugated phenolics

Fig. 3.4.9 is an HPLC chromatogram of the water soluble conjugated phenolics and the radioactivity of the eluate. It is understandable that the radioactivity of Fr-8 and 14 which correspond to caffeoyl and 3,4-dimethoxycinnamoyl glycoside was low. This could be ^{partially accounted for by} the removal of one of [3 H] from the precursor. It is interesting to note the radioactivity of Fr-9. This compound was not detected using HPLC, which indicates that it is present at low concentration. This compound was labelled even after 1.0min. ^{which possibly shows that it is biosynthetically close} to p-coumaric acid. ^{or rapidly synthesized from} The level of radioactivity subsequently increased until 60min. then decreased. This suggests that the compound in Fr-9 is an active intermediate, possibly p-coumaroyl-SCoA which has already been reported as an intermediate in the biosynthesis of phenolics and lignin (Kreuzaler and Hahlbrock, 1972; Zenk, 1979).

Another compound not detectable by HPLC which was eluted between 5-6min. (Fr-6) was also labelled. The radioactivity in Fr-6 was at a low level over the first 5min., but after 60min., a significant amount of [3 H] was present in this compound, which increased until 360min.

The increase in the radioactivity throughout the experiment may indicate that this compound is unlikely to be an active intermediate. There is a question about the nature of this compound, is it a regular metabolite in fruits or an artefact produced from the added p-coumaric acid? However, a longer period of labelling is required to confirm the role of this compound in phenylpropanoid metabolism.

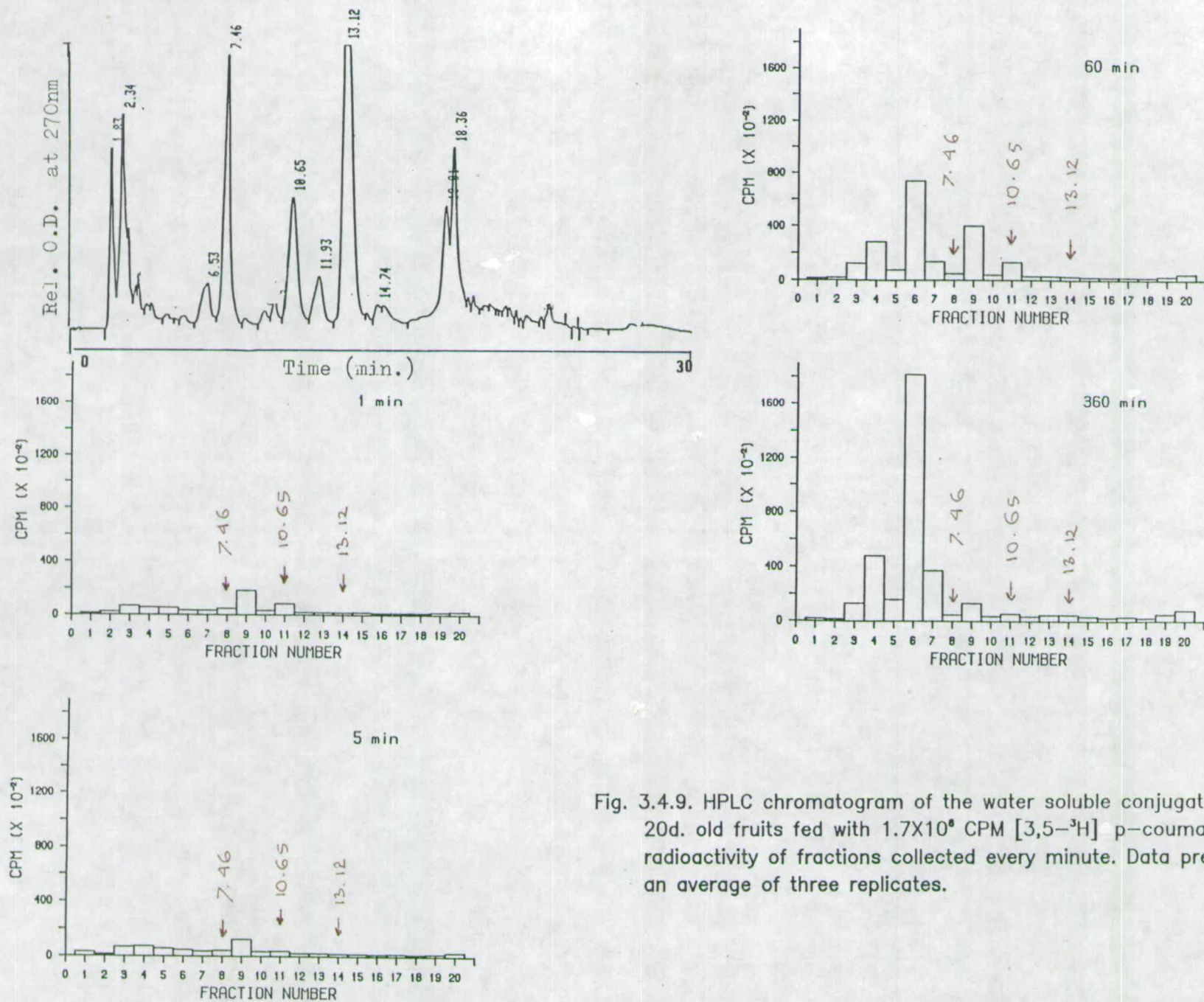


Fig. 3.4.9. HPLC chromatogram of the water soluble conjugated phenolics of 20d. old fruits fed with 1.7×10^6 CPM [3,5- ^3H] p-coumaric acid and the radioactivity of fractions collected every minute. Data presented are an average of three replicates.

3.4.3.2. Time course labelling with [U-¹⁴C] p-coumaric acid

Three questions arose from the previous experiment in which fruits were labelled with p-coumaric acid. 1. Why is [3,5-³H] p-coumaric acid poorly incorporated into capsaicin? 2. What is the role of phenolic-E in capsaicin biosynthesis? 3. What is the role of Fr-6 in phenylpropanoid metabolism? Apart from these questions, further evidence to negate the role of hydroxycinnamoyl glycosides as direct intermediates in phenylpropanoid metabolism is also required. The approach to answering these questions is to use a high SA [U-¹⁴C] p-coumaric acid (synthesized according to the method described in 2.5.4.5). Using uniformly [¹⁴C] labelled p-coumaric acid, there should be no significant loss of radioactivity from this precursor as it proceeds along the pathway leading to capsaicin or to other phenolic compounds.

In this experiment, twelve 20d. old attached fruits were each fed with 1.2μCi [U-¹⁴C] p-coumaric acid (SA 460mCi.mmol⁻¹) in a 30μl solution of phosphate buffer. After 5min., 1, 6 and 24h. three fruits were harvested and the phenolics in each fruits extracted and fractionated according to the methods described in 2.3.2.1 and the radioactivity in each fraction was measured. But prior to this experiment, it was necessary to ensure that p-coumarate uptake by the fruit was efficient.

i. Improvement in the uptake of p-coumaric acid

In the previous experiment, it was noted that a significant level of [³H] was left in p-coumarate after 6h. which could be due to the slow uptake of precursor from the loculus. It is possible that uptake of p-coumarate is affected by pH because the charge on the anion could impede the passage of this compound across the cell membrane. At a higher pH, the ratio of p-coumaric acid as anion to free acid will be higher than at a lower pH (see Henderson-Hasselbach equation below).

$$\text{pH} = \text{pKa} + \log [\text{p-coumaric}^-]/[\text{p-coumaric acid}]$$

$$[\text{p-coumaric}^-]/[\text{p-coumaric acid}] = 10^{(\text{pH}-\text{pKa})}$$

Note: pKa o and m coumaric acid are 4.6 and 4.2 respectively.
pKa p-coumaric acid is unknown, probably between 4-5.

In this experiment, ten 20d. old fruits were used. Two fruits were each fed with 1.3×10^6 CPM [3,5- ^3H] p-coumaric acid in a 30 μl solution of 0.05M phosphate buffer at pH 5.0, 5.5, 6.0, 6.5 and 7.0. After 2h., the fruits were harvested and the phenolics in each fruit extracted according to the methods described in 2.3.2.1. After the evaporation of the MeOH extract, the residue was taken up in 1.5ml MeOH and 30 μl was applied to the TLC-Silica gel and developed with solvent system I. The spots of p-coumaric acid which were identified by using an authentic marker, were removed from the TLC plate and the radioactivity in each spot measured using LSC (2.3.6.2.iii).

Table 3.4.2 shows the level of radioactivity which remained in p-coumaric acid after a labelling period of 2h. with the precursor dissolved in buffer solution over the pH range. Assuming that any p-coumaric acid which has not been metabolized is mainly present in the loculus, it can be seen that an increase in the uptake was observed as the pH decreased.

Table 3.4.2. The effect of pH on the uptake of p-coumaric acid

[pH]	CPM of p-coumarate remained in the loculus
5.0	133,000 (10.23%)
5.5	137,500 (10.58%)
6.0	194,000 (14.92%)
6.5	226,000 (17.40%)
7.0	259,000 (19.92%)

Note: -Total tracer supplied was 1.3×10^6 CPM

-Data are presented as an average of two replicates

The uptake of p-coumarate from the solution at pH 5.0 and 5.5 was similar, hence 0.01M phosphate buffer at pH 5.5 was used to dissolve the [U- ^{14}C] p-coumaric acid.

ii. Distribution of [^{14}C] in free, water soluble conjugates and saponifiable cell wall phenolics, and lignin

The balance sheet of [^{14}C] in different phenolic fractions in fruits after the addition of $1.2\mu\text{Ci}$ [$\text{U}-^{14}\text{C}$] p-coumaric acid can be seen in Fig. 3.4.10. The radioactivity in free phenolics was very high at 5min. due to the fact that added precursor had not been metabolized. The level decreased rapidly over the first 6h., then more slowly between 6h. and 24h. By the end of the labelling period, 11% of the total [^{14}C] supplied was present in this fraction. The labelling pattern for water soluble conjugated phenolics and lignin was similar but in contrast to that for free phenolics. The radioactivity in these two groups of phenolics increased throughout the labelling period and by the end of the experiment (24h.) 32% and 22% of the total [^{14}C] supplied was present in the water soluble conjugated phenolics and lignin respectively. Labelling of the saponifiable cell wall phenolics was very rapid over the first 1h., then increased slightly until 24h. and by the end of the experiment, 10% of the total [^{14}C] supplied was present in this group of phenolics. The total [^{14}C] recovered after 1h. of labelling was more than 95% but only 80% after 6h. The differences in recovery were possibly due to the removal of the two [^{14}C] in the side chain during the β -oxidation of $\text{C}_6\text{-C}_3$ to produce $\text{C}_6\text{-C}_1$ groups of phenolics.

iii. The incorporation of [$\text{U}-^{14}\text{C}$] p-coumaric acid into free phenolics

Extracts of free phenolics were separated using 2D-TLC on Silica gel with solvent system I for the first direction and n-butanol-isopropanol-ammonia-water (2:6:1:1) for the second direction. By using this system, streaking of the caffeic acid spot which was noticed using solvent system II, was avoided. Caffeic acid moves with the first solvent but remains at the origin with the second solvent. Vanillylamine which is a very polar free phenolic also moves in the second direction. Due to the amphoteric behaviour of vanillylamine, this compound is not extracted into the $\text{EtOAc-Et}_2\text{O}$ phase during the liquid-liquid extraction routinely used, hence for the identification of capsaicin intermediates, the MeOH extract of the fruit was applied directly to the TLC plate. Fig. 3.4.11 shows the TLC autoradiograph of the MeOH extract of a 20d. old fruit fed with $1.2\mu\text{Ci}$ [$\text{U}-^{14}\text{C}$] p-coumaric acid for 1h. Phenolic-E

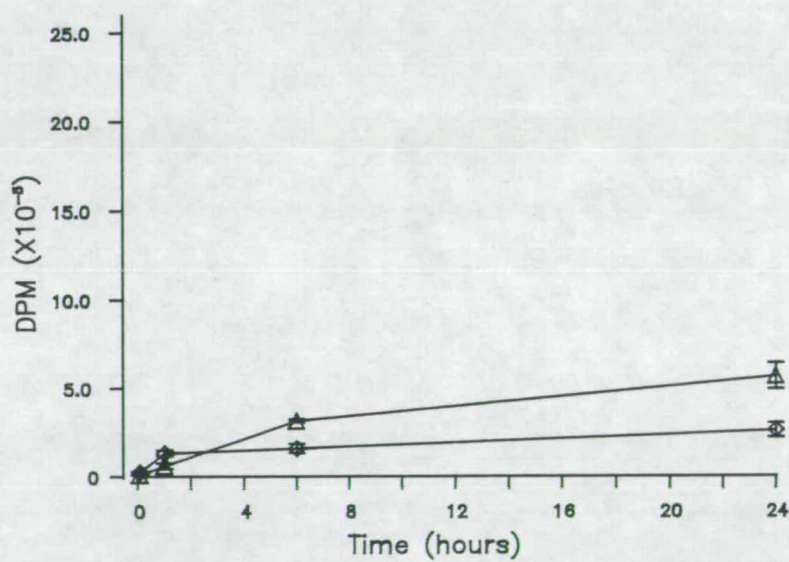
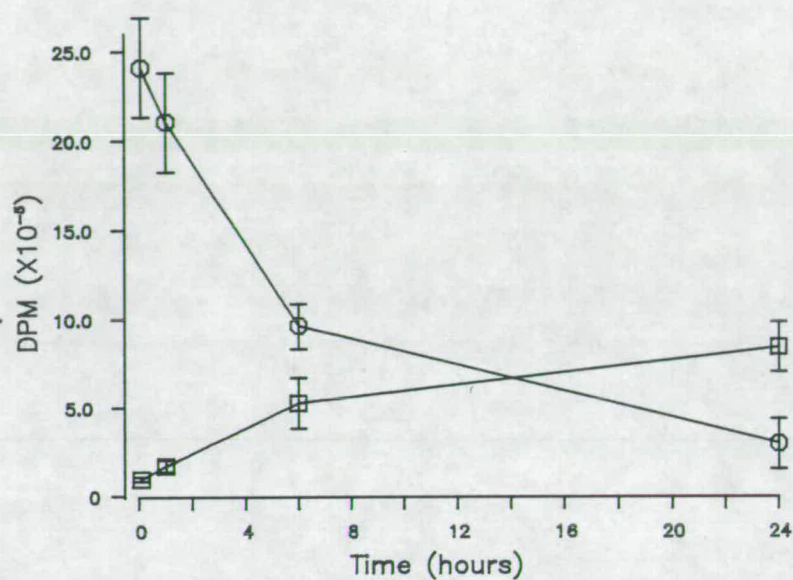


Fig. 3.4.10. Distribution of $[^{14}\text{C}]$ in free phenolics [O], water soluble conjugated phenolics [□], saponifiable cell wall phenolics [◇] and lignin (Δ) in 20d. old fruits fed with $1.2\mu\text{Ci}$ $[\text{U}-^{14}\text{C}]$ p-coumaric acid. Data are presented on *per* fruit basis with bars representing the SE of the mean of three replicates.

was not detected in this experiment, which is consistent with the suggestion made in 3.4.2 that phenolic-E is a detoxification product of added p-coumaric acid. Hydroxycinnamates in this solvent system are separated into *cis* and *trans* isomers. Caffeic and ferulic acid only appeared as very faint spots.

The other capsaicin intermediates, vanillin and vanillylamine were not detected. Two intense spots which did not move in the first direction but separated in the second direction are water soluble conjugated phenolics. In addition, an unknown compound is very radioactive. This compound coelutes with p-coumaric acid in the first direction which indicates the same pattern of substitution in the aromatic ring as that of p-coumaric acid. The higher R_f value of this substance in the second direction may point to the absence of a carboxylic group. However, the high level of radioactivity present suggests that this compound is close to p-coumaric acid in phenylpropanoid metabolism. One of the possibilities is p-coumaraldehyde which is an intermediate in lignin biosynthesis. Further work is required to confirm the identity of this compound.

The change in the radioactivity of the free phenolics during the experiment is presented in Fig. 3.4.12. Radioactivity in p-coumaric acid decreased rapidly within the first 6h. and at the end of the experiment, there was still a low but detectable amount of [¹⁴C] in the precursor. In contrast with previous experiments using [U-¹⁴C]-cinnamic acid, the radioactivity of caffeic and ferulic acid was very low. This could be due to the low uptake of the precursor by the cells. The level of radioactivity in caffeic acid is not significantly different after 5min. and 6h., this may indicate the continuous formation of caffeic acid from p-coumaric acid and rapid metabolism of the caffeic acid produced. Subsequently the level decreased slowly. The radioactivity in ferulic acid which is an intermediate two steps further down than p-coumarate in capsaicin biosynthesis was very low after 5min., then increased until 1h., subsequently the level decreased.

There were significant counts in capsaicin at 5min., subsequently the level of [¹⁴C] in this compound increased steadily until the end of the labelling period.

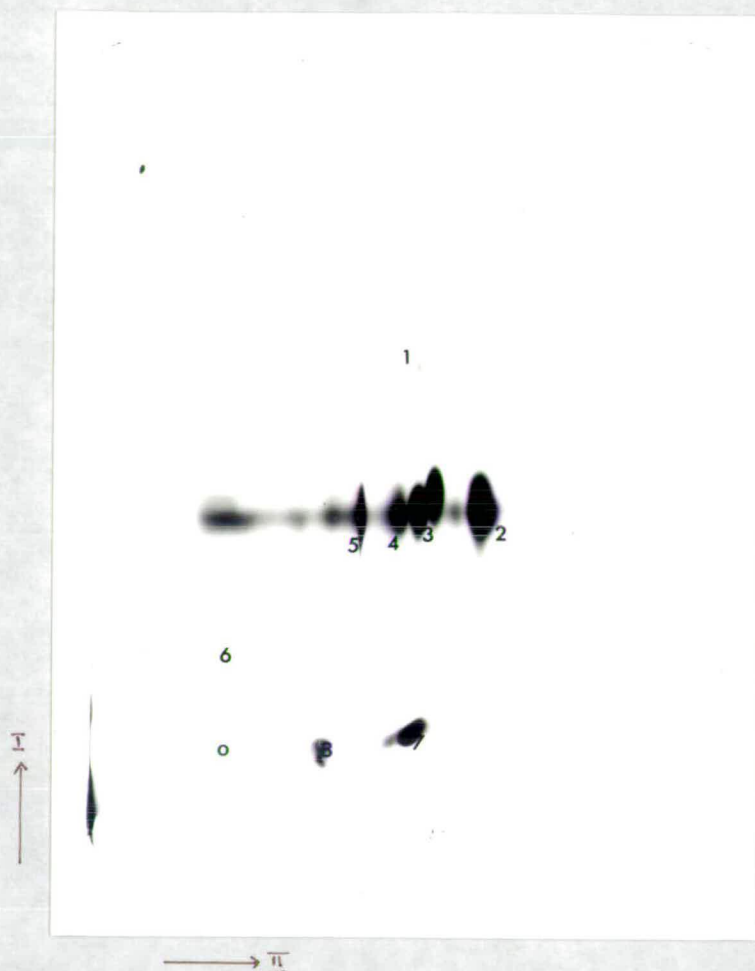


Fig. 3.4.11. 2D-TLC autoradiograph of the MeOH extract of a 20d. old fruit labelled with $1.2\mu\text{Ci}$ $[\text{U}-^{14}\text{C}]$ p-coumaric acid for 1h. Twenty microlitres of a 1.0ml extract was applied. 1. Ferulic acid, 2. possibly p-coumaraldehyde, 3. p-coumaric acid (*cis* and *trans*), 4 and 5. unknown phenolics, 6. caffeic acid, 7 and 8. water soluble conjugated phenolics. O: origin

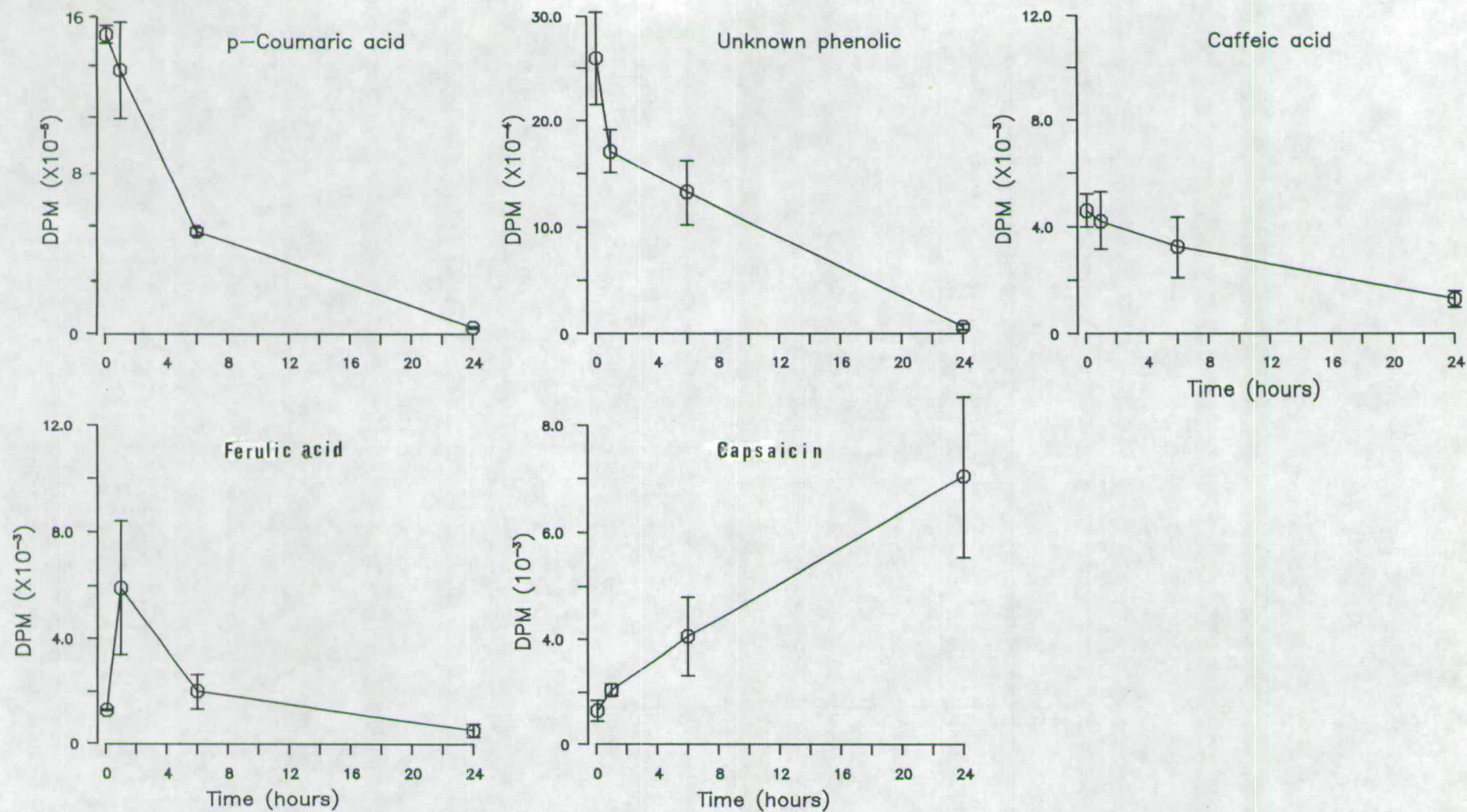


Fig. 3.4.12. Radioactivity of free phenolics in 20d. old fruits fed with $1.2\mu\text{Ci}$ $[\text{U}-^{14}\text{C}]p\text{-coumaric acid}$. Data are presented on *per fruit* basis with bars representing the SE of the mean of three replicates.

Although a higher activity of p-coumaric acid (1.2 μ Ci) was used in this experiment than with cinnamic acid (0.76 μ Ci), lower counts were found in capsaicin. This could be due to the slow uptake of p-coumaric acid. A high activity was present in the unknown compound after 5min., then the amount decreased until the end of the labelling period. The highest count in this compound was at 5min., this was consistent with the suggestion previously made that this compound is formed directly from p-coumaric acid.

iv. Incorporation of [U-¹⁴C] p-coumaric acid into the water soluble conjugated phenolics

Measurement of the radioactivity in water soluble conjugated phenolics was as described in 3.4.3.1. Fig. 3.4.13 shows the labelling pattern of five water soluble conjugated phenolics in 20d. old fruits fed with 1.2 μ Ci [U-¹⁴C] p-coumaric acid. A sequence of labelling was observed with the three hydroxycinnamoyl glycosides. A significant level of radioactivity was detected in p-coumaroyl glycoside at 5min., the level increased until 1h., then decreased slowly. A very low level of radioactivity was present in caffeoyl glycoside at 5min., the level increased until 6h. then decreased slowly. Between 5min. and 1h., the radioactivity in 3,4-dimethoxycinnamoyl glycoside was still very low, then increased slowly until 6h., and subsequently remained constant until the end of the experiment. It is interesting to speculate that the three hydroxycinnamoyl glycosides are interconnected biosynthetically, i.e. p-coumaroyl glycoside \rightarrow caffeoyl glycoside \rightarrow 3,4-dimethoxycinnamoyl glycoside. This possibility was tested in a further experiment. A constant level of radioactivity in 3,4-dimethoxycinnamoyl glycoside after 6h. could be due to the incorporation of [¹⁴C] from the precursor and further metabolism of this compound. However, the possible release of p-coumaric and caffeic acid from their glycoside forms cannot be excluded.

The radioactivity of an unknown compound collected in Fr-6 increased throughout the time course and by the end of the experiment, 25% of the total [¹⁴C] supplied was present in this compound. From this observation, it would seem that this compound is not further metabolized. Also the fact that this compound was not

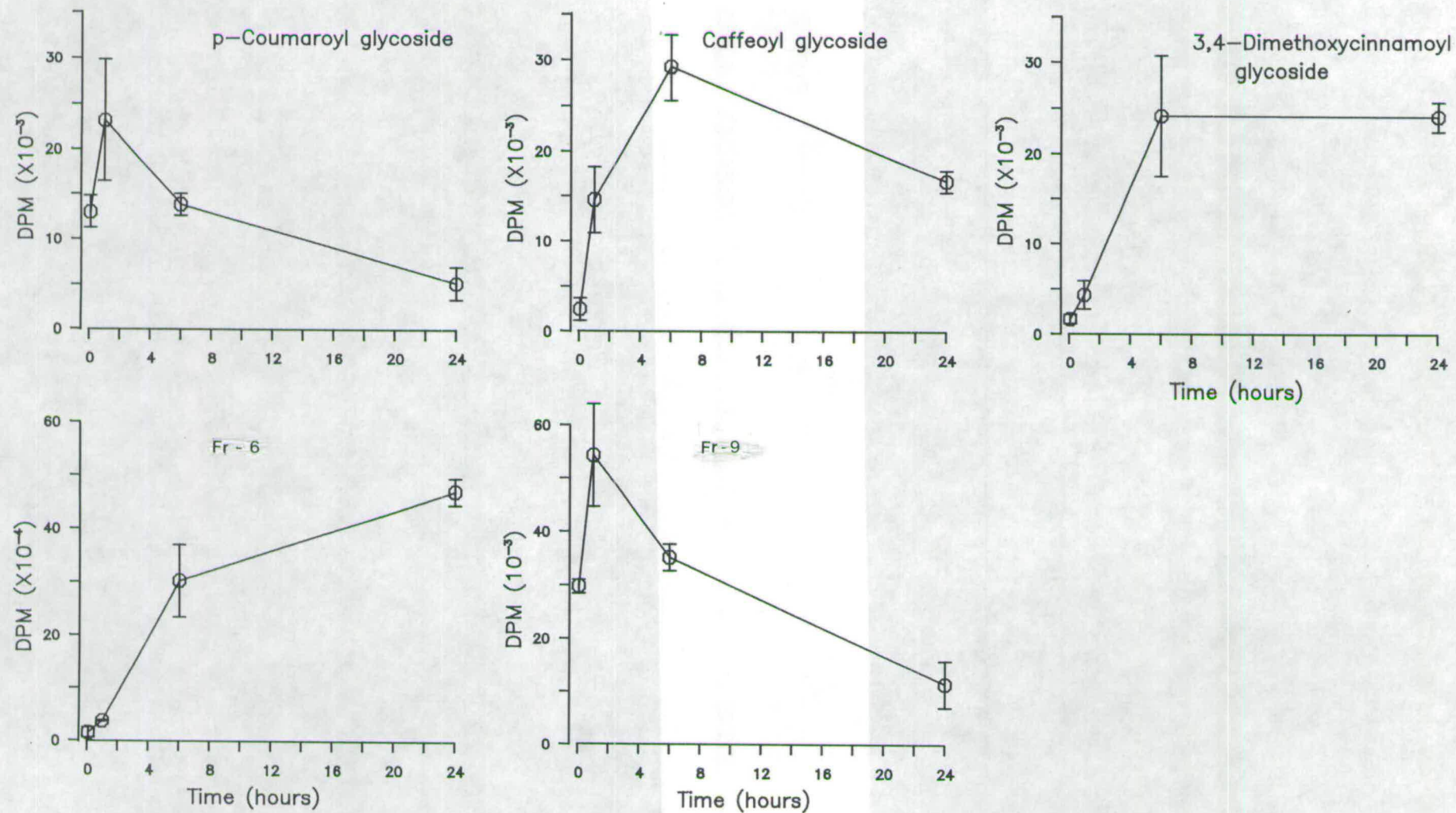


Fig. 3.4.13. Radioactivity of water soluble conjugated phenolics in 20d. old fruits fed with 1.2 μ Ci [U-¹⁴C]p-coumaric acid. Data are presented on *per* fruit basis with bars representing the SE of the mean of three replicates.

✓ However, the fact that most [^{14}C]capsaicin was made during the first 40min. after feeding i.e. before [^{14}C] in ferulic acid started to diminish (see Fig. 3.4.3 in p.119), generates a question about the role of ferulic acid as an active intermediate in capsaicin biosynthesis.

labelled to any significant extent in the experiment with [U-¹⁴C] cinnamic acid would support the idea that this compound is not a "regular" metabolite in fruits.

In this experiment, Fr-9 was rapidly labelled which is consistent with the results from the previous experiment using [3,5-³H] p-coumaric acid. The pattern of labelling of this compound was similar to that of p-coumaroyl glycoside but the level of radioactivity was always higher in this compound. This may suggest that Fr-9 is a more active intermediate than p-coumaroyl glycoside. This compound may be p-coumaroyl-SCoA (see 3.4.3.1).

3.4.4. Summary

p-Coumaric, caffeic and ferulic acid and four unknown free phenolics were all rapidly labelled with [¹⁴C] in 15d. old fruits fed with [U-¹⁴C] cinnamic acid. ^{After 60 min} the label disappeared rapidly in all of these compounds and was paralleled by a ^{continuous} increase in radioactivity in capsaicin. p-Coumaroyl, caffeoyl and 3,4-dimethoxycinnamoyl glycosides also became radioactive soon after the addition of [U-¹⁴C] cinnamic acid, but this radioactivity did not decrease during the experiment after reaching a maximum level. These results are compatible with the proposed pathway of capsaicin biosynthesis. ✓

p-Coumaric and caffeic acid seem to be very toxic towards the cells of pepper fruits. p-Coumaric acid (0.56μmol=91.8μg) added into the fruits was rapidly detoxified by conversion into phenolic-E which was subsequently incorporated into the cell wall. Browning was observed in a 20d. old fruit fed with 0.17μmol (30μg) caffeic acid, and this compound was rapidly converted into MeOH insoluble materials. Detoxification of these two compounds appeared to be taken place in the cell walls.

In 20d. old fruits, the synthesis and further metabolism of the hydroxycinnamoyl glycosides was noted in labelling with high SA [U-¹⁴C] p-coumaric acid. There is the possibility of interconversion between these three glycosides. However, the release of the hydroxycinnamates from these glycosides which may then act as precursors for

the synthesis of capsaicin and lignin cannot be excluded. These possibilities were investigated in 3.5 and 3.6. An active intermediate which was thought to be p-coumaroyl-SCoA was detected in the experiments with [3,5-³H] p-coumaric acid and [U-¹⁴C] p-coumaric acid.

Although p-coumaric acid is a closer intermediate to capsaicin than cinnamic acid, added p-coumaric acid is less efficiently incorporated into capsaicin than cinnamic acid.

One of the possible reason is the diversion of added p-coumaric acid on its way to the site of capsaicin synthesis, shown by the formation of a highly radioactive water soluble conjugated phenolic which did not appear to be further metabolized and seems to be an "irregular" metabolite.

There was no indication that saponifiable cell wall phenolics in chilli pepper fruits are further metabolized. Lignin which is actively synthesized at the same time as that of capsaicin seem to be a major sink in phenylpropanoid metabolism in chilli pepper fruits.

Section 3.5. Synthesis of Hydroxycinnamoyl glycosides in Fruits

3.5. Synthesis of Hydroxycinnamoyl glycosides in Fruits

The synthesis and metabolism of the p-coumaroyl, caffeoyl and 3,4-dimethoxy cinnamoyl glycosides was observed in fruits of stage II (see 3.2 and 3.4). During further metabolism, these compounds probably release phenolic moieties which may then act as intermediates in capsaicin and lignin biosynthesis. Previously (3.3), it has been shown that [U-¹⁴C] cinnamic acid was rapidly incorporated into these compounds in fruits of stage I but no significant radioactivity was present in the free phenolics of fruits at this stage^{after 6h. feeding}. These results suggest that p-coumaric and caffeic acid may not be the direct precursors of p-coumaroyl and caffeoyl glycoside respectively. ^{or these compounds are synthesized rapidly from cinnamic acid.} It is possible that the three hydroxycinnamoyl glycosides in fruits, might be interconnected biosynthetically. Indeed the role of glucose esters of cinnamic acid as intermediates in phenylpropanoid metabolism has already been reported (Molderez *et al.*, 1978; Villegas and Kojima, 1986 and the references therein).

In order to begin to understand this pattern of results, a closer view of the biosynthesis of the hydroxycinnamoyl glycosides is required. It was anticipated that a time course study of the labelling of hydroxycinnamoyl glycosides in fruits of stage I (d.10) with [U-¹⁴C] cinnamic acid and [U-¹⁴C] p-coumaric acid, would reveal the nature of the intermediates to these compounds. All of these experiments were carried out using 10d. old fruits (stage I) which incorporated [U-¹⁴C] cinnamic acid mainly into the hydroxycinnmoyl glycosides (3.3). In addition, labelling with [2-side chain-¹⁴C] p-coumaric and caffeic acid was also carried out to determine which phenolic is more likely to be the intermediate of the caffeoyl glycoside.

3.5.1. Time Course Labelling with [U-¹⁴C] cinnamic acid

In this experiment, 18 attached fruits were each fed with 0.76μCi [U-¹⁴C] cinnamic acid [SA=460mCi.mmol⁻¹] in a 30μl solution of 0.05M phosphate buffer at pH 7.0. After 10, 20, 40, 60, 180 and 360 min., three fruits were removed from the plants and the phenolics in each fruit extracted and fractionated according to

the methods described in 2.3.2.1.

3.5.1.1. Distribution of [^{14}C] in free and water soluble conjugated phenolics

Fig. 3.5.1. shows that [$\text{U-}^{14}\text{C}$] cinnamic acid was rapidly incorporated into the water soluble conjugated phenolics. Within 10min., 37% of [^{14}C] supplied was present in these compounds. The level increased rapidly until 60min. then remained constant at 85% of total [^{14}C] supplied. The radioactivity in the free phenolic fraction was high at 10min. due to the presence of [$\text{U-}^{14}\text{C}$] cinnamic acid which had not been metabolized. The level then decreased rapidly over the first 60min. and by the end of the labelling period, only 2% of [^{14}C] supplied was present in this fraction. These results show that the water soluble conjugated phenolics are likely to be derived more or less directly from cinnamic acid.

3.5.1.2. [^{14}C] in cinnamic, p-coumaric, caffeic and ferulic acid

The level of [^{14}C] in p-coumaric, caffeic and ferulic acid was also measured in order to investigate the role of these compounds in the synthesis of the hydroxycinnamoyl glycosides. To measure the radioactivity in these phenolics, 20 μl of the free phenolic extract (1.0ml) was applied to a TLC-Silica gel and developed with solvent system I. Prior to this development 10 μl of a solution containing a mixture of cinnamic, p-coumaric, caffeic and ferulic acid in MeOH (1.0 mg.ml $^{-1}$ respectively) was also loaded onto the spots of the extracts to facilitate identification. After development, the spots of the corresponding compounds were scraped off and the radioactivity counted using LSC (2.3.6.2.iii).

Fig. 3.5.2 shows the radioactivity in cinnamic, p-coumaric acid, caffeic and ferulic acid over the time course. The radioactivity in cinnamic acid was high at 10min., however, the level decreased rapidly over the first 60min. and had reached a very low level by the end of the experiment. A significant level of [^{14}C] (73,000 DPM) was observed in p-coumaric acid at 10min., this increased and reached a maximum level at 20min. (114,000 DPM), then decreased rapidly. As with cinnamic acid, there was only a very small amount of [^{14}C] in this compound by the end of the

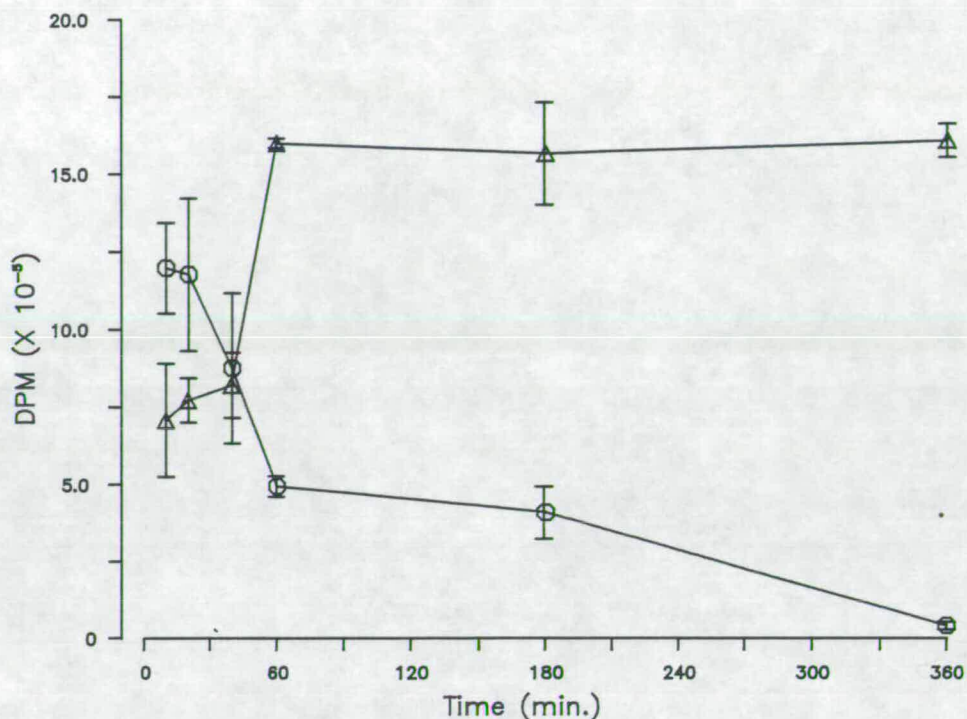


Fig. 3.5.1. Changes in the radioactivity in free [O] and water soluble conjugated phenolics [Δ] during time course labelling of phenolics in 10d. old fruits with $0.76\mu\text{Ci}$ [$\text{U-}^{14}\text{C}$] cinnamic acid. Data are presented on a *per fruit* basis with bars representing the SE of the mean of three replicates.

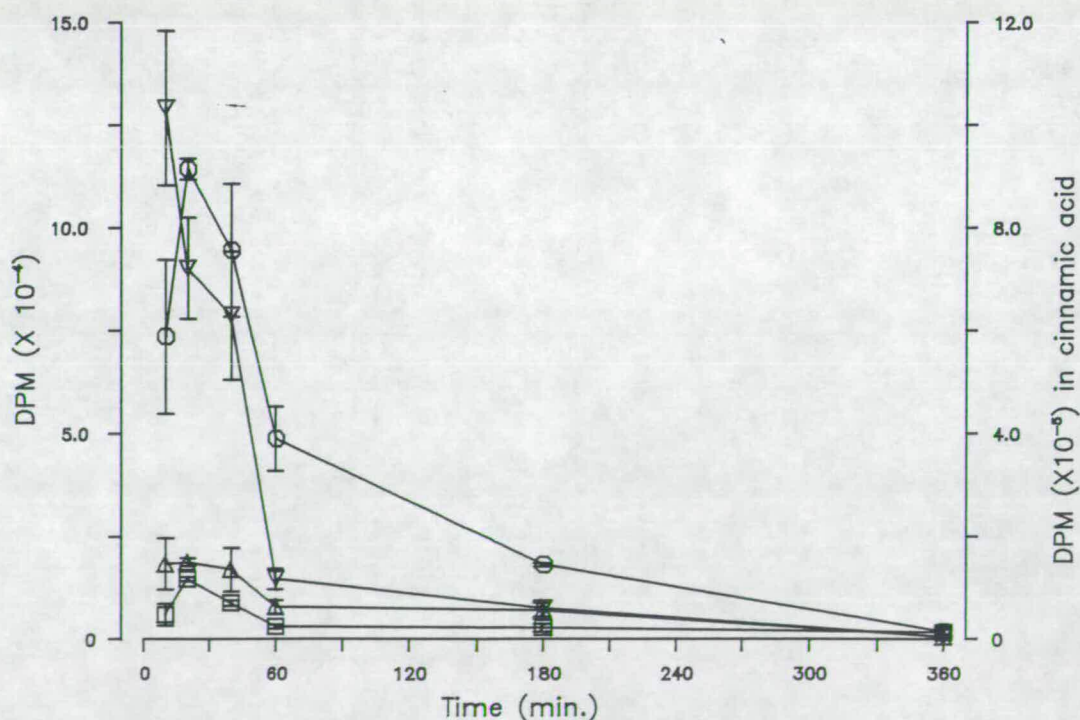


Fig. 3.5.2. Changes in the radioactivity in p-coumaric [O], caffeic [Δ], ferulic [□] and cinnamic acid [▽] during time course labelling of phenolics in 10d. old fruits with $0.76\mu\text{Ci}$ [$\text{U-}^{14}\text{C}$] cinnamic acid. Data are presented on a *per fruit* basis with bars representing the SE of the mean of three replicates.

experiment. These results show that cinnamic and p-coumaric acid are being actively synthesized and further metabolized in fruits of this stage.

Caffeic and ferulic acid were both labelled, but the radioactivity in these compounds was very low. The maximum levels of [^{14}C] in caffeic and ferulic acid were both reached at 20min., but the amounts were only 19,000 and 15,000 DPM respectively. Since 85% of the [^{14}C] supplied was present in the water soluble conjugated phenolics, there is no doubt that p-coumaric acid is an intermediate of these compounds in 10d. old fruits. However, it is questionable whether or not caffeic and ferulic acid are the immediate precursors of caffeoyl and 3,4-dimethoxycinnamoyl glycoside respectively. The low levels of caffeic and ferulic acid observed in fruits of this stage might be used for the synthesis of lignin and saponifiable cell wall phenolics, as a low incorporation of [$\text{U-}^{14}\text{C}$] cinnamic acid was present in these compounds (3.3).

3.5.1.3. [^{14}C] in the water soluble conjugated phenolics

Separation of the water soluble conjugated phenolics and measurement of their radioactivity was as described in 3.4.3.1. The changes in radioactivity in p-coumaroyl, caffeoyl and 3,4-dimethoxycinnamoyl glycoside and Fr-9 (described in 3.4.3) were followed over the time course (see Fig. 3.5.3). The radioactivity in Fr-6 was very low. The level of [^{14}C] in p-coumaroyl glycoside was low over the first 20min. increased to a maximum level at 180min., and then remained constant. [$\text{U-}^{14}\text{C}$] Cinnamic acid was rapidly incorporated into caffeoyl and 3,4-dimethoxycinnamoyl glycosides. A significant level of [^{14}C] was noted in both compounds at 10min., then the amount increased^{less} rapidly until 60min. The radioactivity in caffeoyl glycoside remained constant after 60min. until the end of the experiment, in contrast to 3,4-dimethoxycinnamoyl glycoside in which the counts increased after 180min. A significant level of radioactivity was also noted in Fr-9 at 10min. The level of [^{14}C] in this compound increased until 60min. and then decreased. The incorporation pattern of [$\text{U-}^{14}\text{C}$] cinnamic acid into Fr-9 was in agreement with the previous experiment (see 3.4.3.1 and 3.4.3.2). It is very likely that

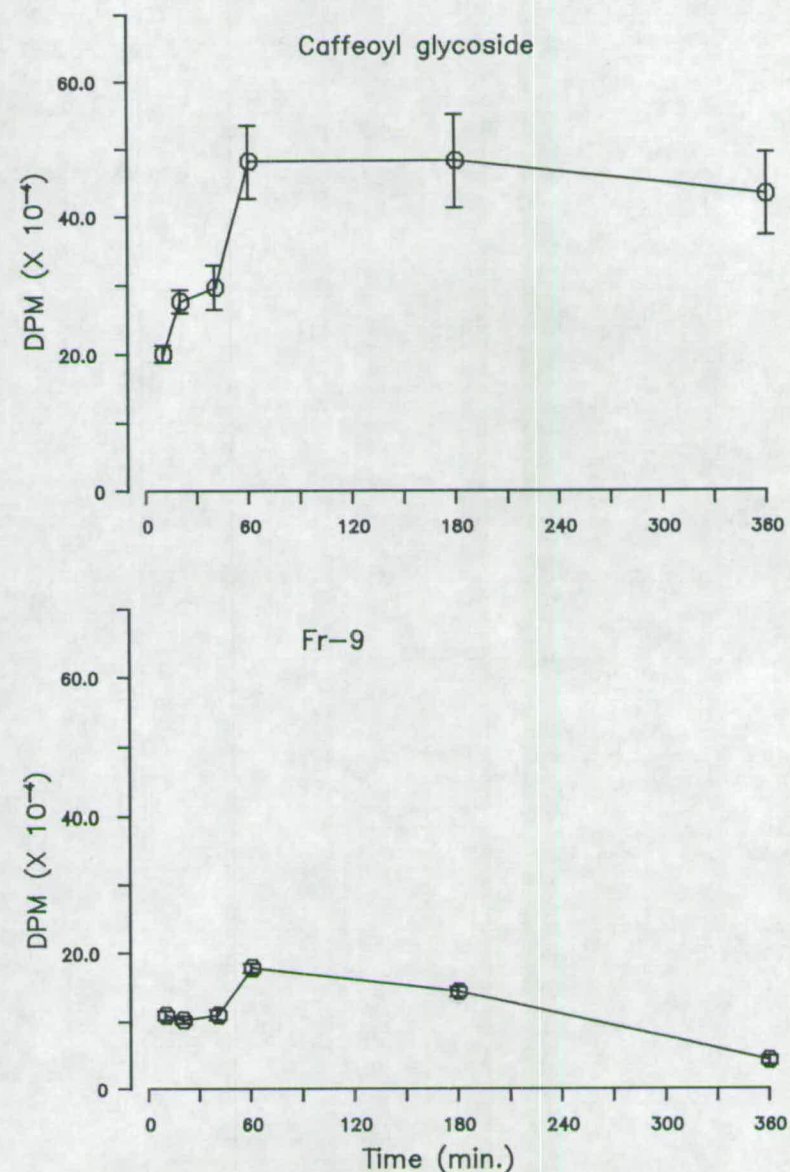
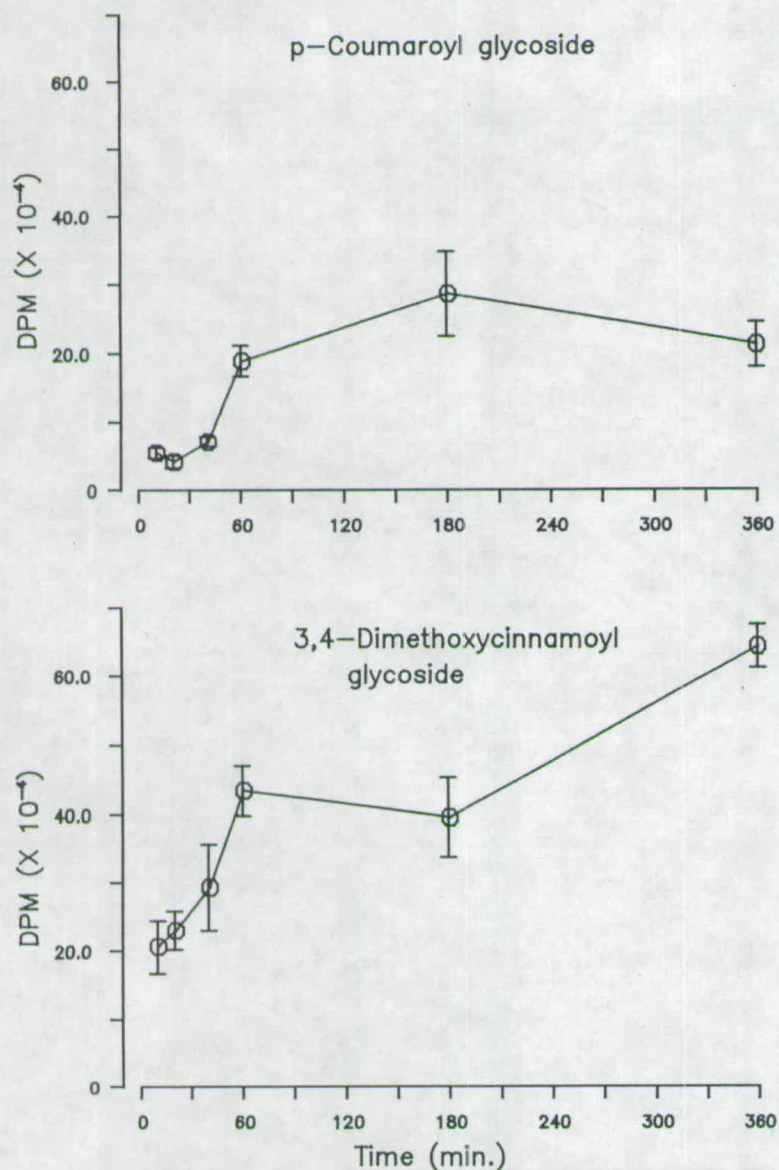


Fig. 3.5.3. Changes in the radioactivity in specific compounds of the water soluble conjugated phenolics during time course labelling of phenolics in 10d. old fruits with $0.76\mu\text{Ci}$ $[\text{U-}^{14}\text{C}]$ cinnamic acid. Data are presented on a *per fruit* basis with bars representing the SE of the mean of three replicates.

this compound is an active intermediate in phenylpropanoid metabolism in fruits.

3.5.2. Time Course Labelling with [U-¹⁴C] p-Coumaric acid

It was established in 3.5.1 that p-coumaric acid or a close derivative is an intermediate between cinnamic acid and ^{at least certain pools of} Δ hydroxycinnamoyl glycosides, however, its nature is still uncertain. It might be expected that by applying a rapid pulse of [U-¹⁴C] p-coumaric acid, the nature of the intermediate immediately after p-coumaric acid would be revealed. In this experiment, 12 attached fruits were each fed with 0.78 μ Ci [U-¹⁴C] p-coumaric acid (SA 460mCi.mmol⁻¹) in a 20 μ l solution of 0.01M phosphate buffer at pH 5.5. Three fruits were removed from the plant at 5min., 1, 6 and 24h., and the phenolics in each fruit extracted and fractionated according to the methods described in 2.3.2.1.

3.5.2.1. Distribution of [¹⁴C] in free and water soluble conjugated phenolics (Fig. 3.5.4)

The data presented in Fig. 3.5.4. show that the radioactivity in free phenolics decreased rapidly over the first 6h. but there was still a significant level of [¹⁴C] after 24h. Compared with the labelling pattern obtained with [U-¹⁴C] cinnamic acid, the decrease in radioactivity in the free phenolic fraction was much slower. As previously (3.5.1.1), the decrease in the radioactivity of free phenolics coincided with the increase in incorporation of precursor into the water soluble conjugated phenolics.

3.5.2.2. [¹⁴C] in p-coumaric, caffeic and ferulic acid (Fig. 3.5.5)

The radioactivity in p-coumaric acid decreased rapidly over the first 6h but was still at a detectable level after 24h. However, the radioactivity present in caffeic and ferulic acid in this case was lower than in the experiment with [U-¹⁴C] cinnamic acid. These results, however, do not exclude the possibility that these compounds act as intermediates in the synthesis of caffeoyl and 3,4-dimethoxycinnamoyl glycoside, because a similar result was also observed in the labelling of phenolics in 20d. old fruits (stage II) ^{Stage II fruits also} incorporated a high level of [U-¹⁴C] cinnamic acid into free with [U-¹⁴C]p-coumaric acid.

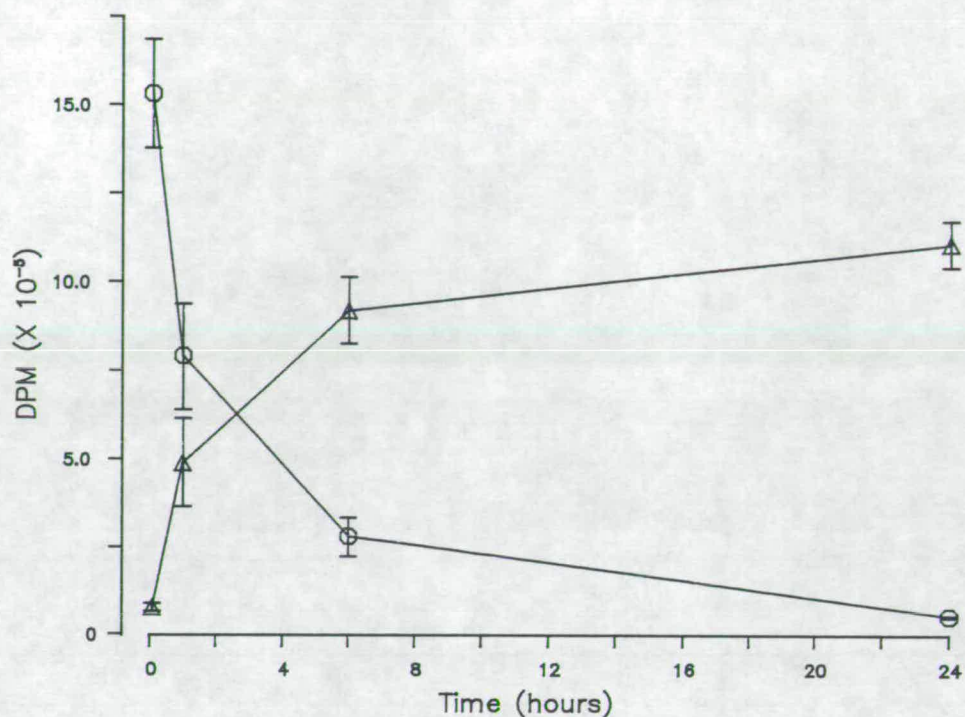


Fig. 3.5.4. Changes in the radioactivity in free [O] and water soluble conjugated phenolics [Δ] during time course labelling of phenolics in 10d. old fruits with $0.78\mu\text{Ci}$ [$\text{U-}^{14}\text{C}$] p-coumaric acid. Data are presented on a *per fruit* basis with bars representing the SE of the mean of three replicates.

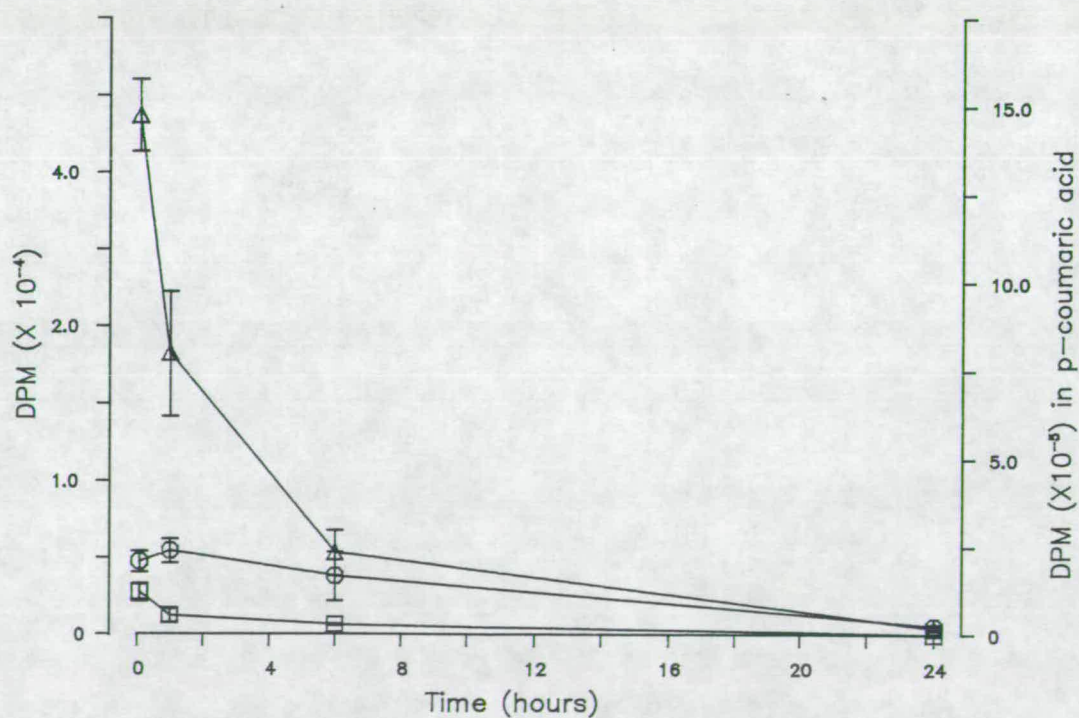


Fig. 3.5.5. Changes in the radioactivity in p-coumaric [Δ], caffeic [O], and ferulic acid [□] during time course labelling of phenolics in 10d. old fruits with $0.78\mu\text{Ci}$ [$\text{U-}^{14}\text{C}$] p-coumaric acid. Data are presented on a *per fruit* basis with bars representing the SE of the mean of three replicates.

phenolics including ferulic acid. This pattern of results appeared to be due to the slow uptake of p-coumaric acid from the locus.

3.5.2.3. [^{14}C] in water soluble conjugated phenolics (Fig. 3.5.6)

The results in Fig. 3.5.6 are consistent with previous results (3.4.3 and 3.5.1) which also show that Fr-9 was rapidly labelled. Within 5min. a significant level of radioactivity was observed in this compound. The level then increased until 60min. remained constant between 1 and 6h. and then decreased. Although the level of [^{14}C] in Fr-9 was lower than in the hydroxycinnamoyl glycoside, it was much higher than in caffeic and ferulic acid. It was also noted that a high count in Fr-9 (4×10^5 DPM) was present in one of the replicates sampled at 1h.

Incorporation of [^{14}C] was also noted in p-coumaroyl glycoside at 5min. which increased until 6h. then remained constant. The level of [^{14}C] in caffeoyl glycoside was very low at 5min., then a pattern of labelling similar to p-coumaroyl glycoside was observed. The labelling pattern of 3,4-dimethoxycinnamoyl glycoside and Fr-6 was similar. The radioactivity in both compounds was very low at 5min. then increased until the end of the experiment.

In addition to a slower incorporation of [$\text{U-}^{14}\text{C}$] p-coumaric acid into the hydroxycinnamoyl glycosides than the [$\text{U-}^{14}\text{C}$] cinnamic acid, the overall level of incorporation was also lower. The low incorporation of [$\text{U-}^{14}\text{C}$] p-coumaric acid into the hydroxycinnamoyl glycosides could be due to the metabolism of p-coumaric acid on its way to the site of central phenylpropanoid metabolism. One of the proposed products of this metabolism is Fr-6. The radioactivity present in this compound was 37.5% of the total [^{14}C] supplied. This compound is possibly an artefact (see 3.4.3).

The lower level of [^{14}C] in Fr-9 observed in this experiment than in the experiment with [$\text{U-}^{14}\text{C}$] cinnamic acid, was consistent with the lower incorporation of [$\text{U-}^{14}\text{C}$] p-coumaric acid into the hydroxycinnamoyl glycosides. It is interesting to speculate that this compound is an intermediate between p-coumaric acid and

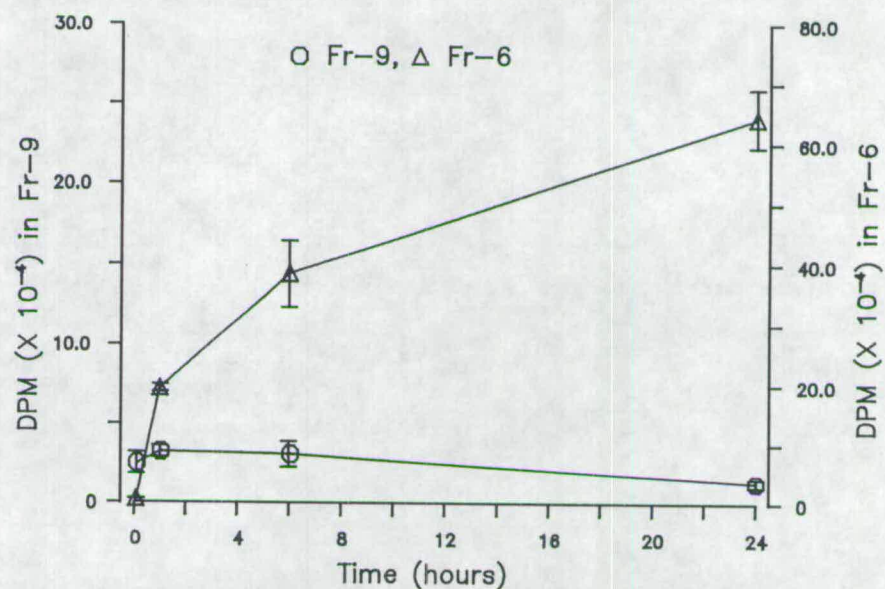
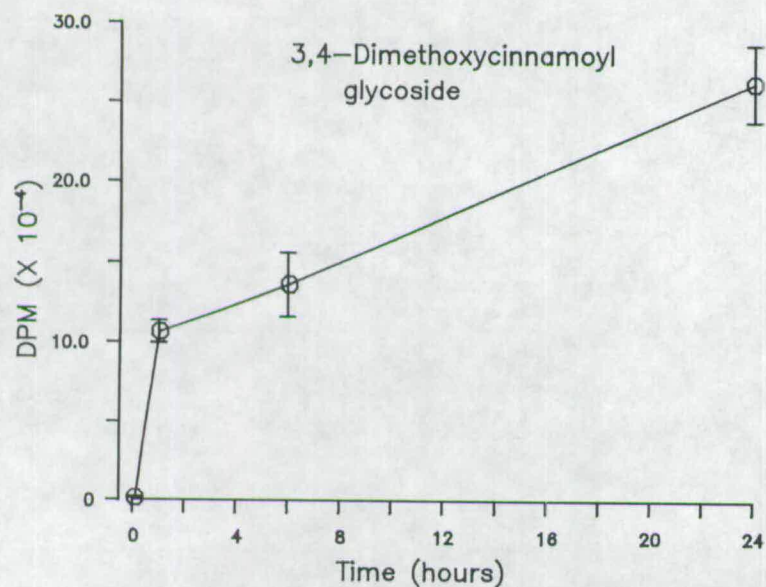
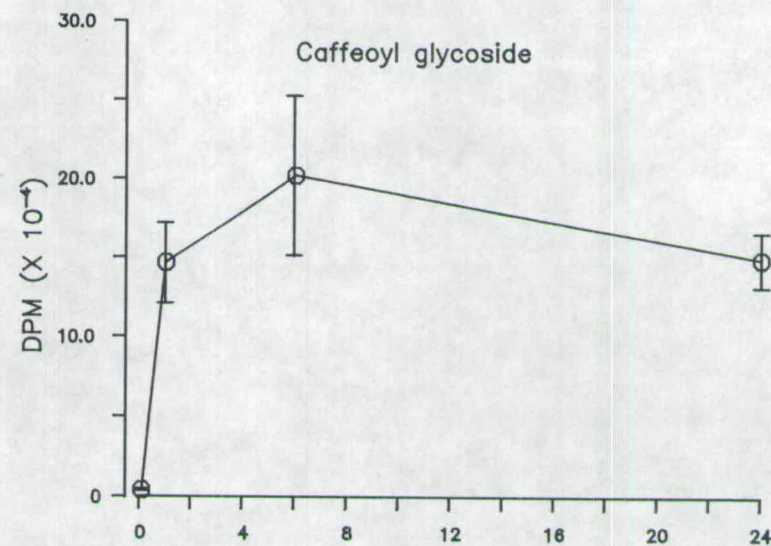
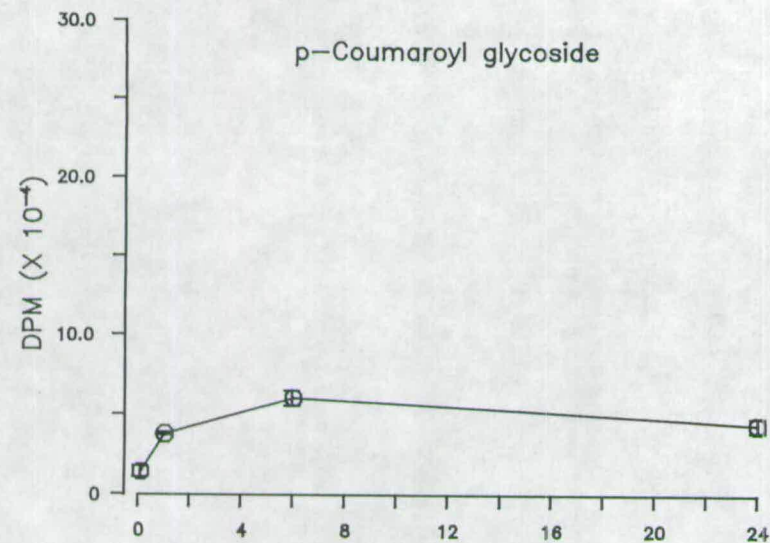


Fig. 3.5.6. Changes in the radioactivity in specific compounds of the water soluble conjugated phenolics during time course labelling of phenolics in 10d. old fruits with $0.78\mu\text{Ci}$ [$\text{U-}^{14}\text{C}$] p-coumaric acid. Data are presented on a *per fruit* basis with bars representing the SE of the mean of three replicates.

p-coumaroyl glycoside. So far there is no evidence to reject the following sequence of reactions:

p-Coumaroyl glycoside → caffeoyl glycoside → 3,4-dimethoxycinnamoyl glycoside

However, further work is needed to establish this sequence. A detailed structure of the hydroxycinnamoyl glycosides is required together with additional biosynthetic studies.

3.5.3. Comparative labelling with [2-side chain- ^{14}C] p-coumaric acid and [2-side chain- ^{14}C] caffeic acid

It has been shown that p-coumaric acid is an intermediate in the synthesis of ^{at least certain pools of} $\sqrt{\text{some}}$ caffeoyl glycoside, and it has been speculated that this compound is synthesized via p-coumaroyl glycoside. However, there is no strong evidence to eliminate the possibility that caffeic acid acts as a precursor for caffeoyl glycoside. Although the level of [^{14}C] in caffeic acid after labelling with [$\text{U-}^{14}\text{C}$] cinnamic acid and [$\text{U-}^{14}\text{C}$] p-coumaric acid was very low, it may well reflect the small pool size of caffeic acid and the rapid conversion of caffeic acid into caffeoyl glycoside.

In this experiment, two sets of 6 fruits were used. The first set (6 fruits) was each fed with 0.5×10^6 DPM [2-side chain- ^{14}C] p-coumaric acid (SA $2.5 \text{ mCi.mmol}^{-1}$) and the second with [2-side chain- ^{14}C] caffeic acid of the same activity and the same SA. Both precursors were delivered in a $30 \mu\text{l}$ solution of 0.05M phosphate buffer at pH 7.0. After a 6h. labelling period, the phenolics in each fruit were extracted and fractionated. Free and water soluble conjugated phenolics were separated by TLC and detected by autoradiography.

Browning of tissue, especially on the surface of seeds was noted during the extraction of fruits fed with [2-side chain- ^{14}C] caffeic acid. No radioactive spots were detected on the chromatograms of either free or water soluble conjugated phenolics. Probably the caffeic acid fed was oxidized or polymerized to produce MeOH insoluble materials.

In fruits fed with [2-side chain- ^{14}C] p-coumaric acid, there was no obvious effect of the precursor but an intense radioactive spot of free phenolic- E (described in Fig. 3.4.2) was observed. The level of [^{14}C] left as p-coumaric acid was very low after 7h. Radioactive spots of caffeoyl and 3,4-dimethoxycinnamoyl glycoside were also detected. Other radioactive spots on the TLC-autoradiograph of the water soluble conjugated phenolic extracts were also observed.

Due to the toxic effect of caffeic acid, no conclusions can be drawn from this experiment. A different approach will have to be employed in further studies. A higher SA of radioactive caffeic acid is required to avoid this toxic effect. Since caffeic acid is more polar than p-coumaric acid, it may be taken up more slowly by the cells lining the loculus.

3.5.4. Summary

p-Coumaric acid has been shown to be an intermediate in the synthesis of ^{at least certain pools of} V ^{some} p-coumaroyl, caffeoyl and 3,4-dimethoxycinnamoyl glycosides. A water soluble conjugated phenolic which on HPLC eluted between 8 and 9 min. (Fr-9) also appeared to be an active intermediate. This compound was tentatively identified as p-coumaroyl-SCoA. As with the labelling of phenolics in 20d. old fruits with [$\text{U-}^{14}\text{C}$] p-coumaric acid, in these experiments, a large proportion of [$\text{U-}^{14}\text{C}$] p-coumaric acid was also incorporated into an unknown water soluble conjugated phenolic (Fr-6) which did not appear to be further metabolized. The level of radioactivity in caffeic and ferulic acid in the experiments with [$\text{U-}^{14}\text{C}$] cinnamic acid and [$\text{U-}^{14}\text{C}$] p-coumaric acid ^{was very low, therefore} the two compounds may not be the intermediates for caffeoyl and 3,4-dimethoxycinnamoyl glycoside respectively. 3,4-Dimethoxycinnamoyl glycoside is probably synthesized from p-coumaric acid via p-coumaroyl glycoside and caffeoyl glycoside.

Section 3.6.
Metabolic Fate of Hydroxycinnamoyl Glycosides in Fruits

3.6. Metabolic Fate of Hydroxycinnamoyl Glycosides in Fruits

A possible pathway for the synthesis of hydroxycinnamoyl glycosides has been proposed in 3.5.4. These compounds which are actively synthesized and accumulated in fruits of stage I, disappear in the later stages of development (see 3.2 and 3.3). The aim of the experiments reported in this section was to investigate the metabolic fate of these compounds using a long period of labelling of 10d. old fruits with [U-¹⁴C] cinnamic acid. Fruits at this stage have already been shown to incorporate [U-¹⁴C] cinnamic acid mainly into the hydroxycinnamoyl glycosides and will presumably, during an extended labelling period, convert these glycosides into products which will be identified. An attempt was made to uncover the mechanism involved in the catabolism of hydroxycinnamoyl glycosides by investigating the enzyme(s) involved in the hydrolysis of these glycosides.

3.6.1. A long period of labelling with [U-¹⁴C] cinnamic acid

This experiment was designed to identify the metabolic fate of the hydroxycinnamoyl glycosides. In this experiment, two attached 10d. old fruits were each fed with 1.0 μ Ci of [U-¹⁴C] cinnamic acid [SA=460mCi.mmol⁻¹] in 40 μ l of 0.05M phosphate buffer at pH 7.0, then left for 25d. The aliquot of labelled cinnamate was injected as two amounts of 20 μ l with an interval of 6h. to avoid any injury which may be caused by the addition of a large volume of liquid into a small fruit (5cm length). After 25d., it was noted that one of the fruits had already turned red while the other remained green, at which stage the fruits were removed from the plant and the phenolics in each fruit extracted and fractionated according to the methods described in 2.3.2.1. The extracts of free phenolics from each fruit were separated by 2D-TLC with solvent system I and II for the first and second direction respectively. The radioactive spots were detected by autoradiography. Fig. 3.6.1. shows the 2D-TLC autoradiographs of free phenolics in the red fruit [A] and in the green fruit [B]. Two spots appeared on both chromatograms. One of the spots was

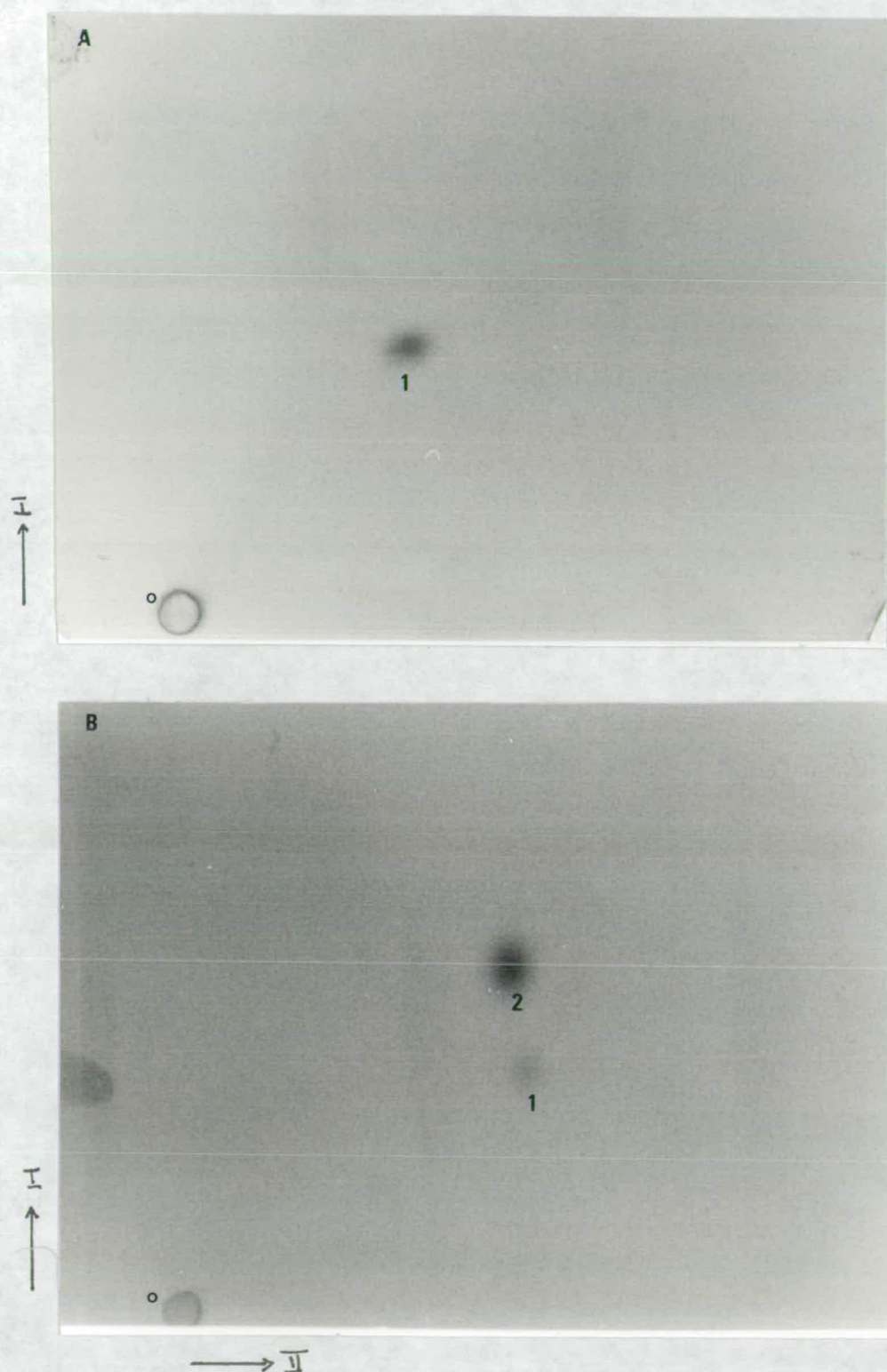


Fig. 3.6.1. 2D-TLC autoradiograph of the free phenolic extracts of 10d. old fruits labelled with $1.0\mu\text{Ci}$ $[\text{U-}^{14}\text{C}]$ cinnamic acid for 25d. TLC was on Silica gel with solvent system I and II for the first and second development respectively. [A] Red fruit, [B] green fruit, [1] capsaicin and [2] possibly 3,4-dimethoxy cinnamic acid.
o: origin

identified as capsaicin and the other is possibly 3,4-dimethoxycinnamic acid. The identity of these two compounds was confirmed by analysing the extracts with HPLC and measuring the radioactivity in the eluate (data not presented). It is interesting to note that a more intense capsaicin spot was present on the 2D-TLC autoradiograph of the red fruit extract while the 3,4-dimethoxycinnamic acid spot was much darker in the green fruit.

The radioactivity in capsaicin, 3,4-dimethoxycinnamic acid, lignin, saponifiable cell wall phenolics and the hydroxycinnamoyl glycosides were all assayed and the results are presented in Table 3.6. The radioactivity of the hydroxycinnamoyl glycosides was determined after separation of the water soluble extracts of conjugates using an HPLC chromatograph and a fraction collector. Hydroxycinnamoyl glycosides in both fruits were not detected by HPLC, but significant counts were still found in the fractions which correspond to the R_t values of these compounds.

Table 3.6.1. The distribution of [^{14}C] in various compounds following 25d labelling of 10d. old fruits with $1.0\mu\text{Ci}$ [$\text{U-}^{14}\text{C}$] cinnamic acid

Compounds	Radioactivity (in 1000 DPM)	
	Green fruit	Red fruit
Capsaicin	26	83
3,4-Dimethoxycinnamic acid	122	9
p- Coumaroylglycoside	6	17
Caffeoylglycoside	65	62
3,4-Dimethoxycinnamoyl glycoside	54	78
Saponifiable cell wall phenolics	312	626
Lignin	1370	1782

The fact that most of the hydroxycinnamoyl glycosides were finally incorporated into lignin and capsaicin was consistent with the location of these compounds in the fruits, here the precursors and products are situated in the same tissue compartment

(see Table 3.2.2). Although it has not been studied here, lignin would seem to be mainly accumulated in the seeds.

3.6.2. Metabolic fate of hydroxycinnamoyl glycosides over a time course

The metabolic fate of the hydroxycinnamoyl glycosides has been identified in 3.6.1. This experiment was designed to elucidate the possible mechanism involved in this metabolism. If these compounds release their phenolic moieties, the free p-coumaric, caffeic and 3,4-dimethoxycinnamic acid should be detected during a time course of metabolism.

In this experiment, 15 attached 10d. old fruits were each fed with 0.76 μ Ci [U- 14 C] cinnamic acid [SA=460mCi.mmol $^{-1}$] in 30 μ l of 0.05M phosphate buffer at pH 7.0. After 6h., 5, 10, 20, and 30d. three fruits were removed and the phenolics in each fruit extracted and fractionated according to the methods described in 2.3.2.1. The radioactivity in free, water soluble conjugated and saponifiable cell wall phenolics and in lignin were all measured. Fig. 3.6.2. shows the changes in the levels of [14 C] in these groups of compounds during a long period of labelling. After 6h., only a small proportion of the radioactivity was present in the free phenolics. The radioactivity in the saponifiable cell wall phenolics was also low and did not change between 6h and 30d. A very high level of [14 C] was found in the water soluble conjugated phenolics after 6h. and the level then decreased until the end of the labelling period. The radioactivity in lignin at 6h. was low and the level only increased slightly between 6h and 10d. A large increase in the level of [14 C] in lignin was observed between 10 and 20d. (correspond to fruits of d.20 and d.30) then the level remained constant.

Separation of the components in the free phenolic extracts by one dimensional TLC with solvent system II followed by autoradiography showed that only capsaicin was labelled. There was no radioactivity in the other free phenolics which is possibly due to the low activity of precursor used. The radioactivity in the hydroxycinnamoyl glycosides was counted after separation of the water soluble conjugated phenolic extracts using an HPLC chromatograph and a fraction collector. Changes in the

Contribution from the radioactivity of water soluble conjugates in free phenolic fraction cannot be avoided and [14 C] in capsaicin only accounts for small proportion. Therefore, the kinetic of incorporation [14 C] into capsaicin will have little effect on the overall picture.

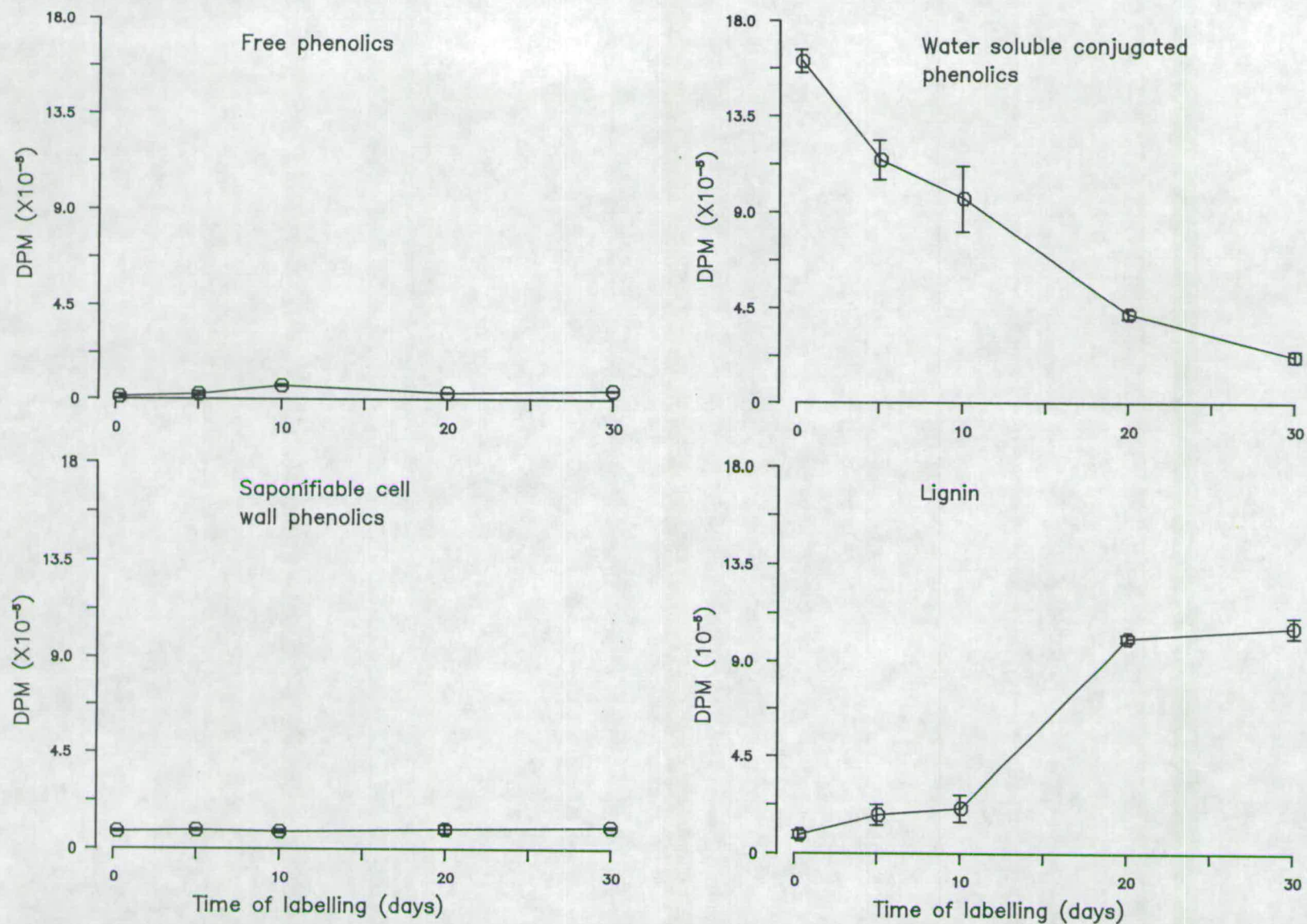


Fig. 3.6.2. Changes in the radioactivity in various groups of compounds during a long period of labelling in 10d. old fruits with 0.76 μ Ci [U-¹⁴C] cinnamic acid. Data are presented on a *per fruit* basis with bars representing the SE of the mean of three replicates.

radioactivity of these compounds are presented in Fig. 3.6.3. A rapid decrease in the radioactivity of p-coumaroyl glycoside was observed between 6h. and 5d. During this period, there was no significant decrease in the radioactivity of caffeoyl glycoside. Subsequently the radioactivity decreased rapidly and only a very small amount of [^{14}C] was present in this compound after 30d. The radioactivity in 3,4-dimethoxycinnamoyl glycoside decreased between 6h. and 20d. then remained constant at 15% of the total radioactivity supplied. The level of [^{14}C] in capsaicin was very low after 6h. but was heavily labelled after 5d. Subsequently the radioactivity in capsaicin increased until the end of the labelling period.

3.6.3. Trapping the free phenolics released by the catabolism of hydroxy cinnamoyl glycosides with cold caffeic and ferulic acid

It has been shown in 3.6.2 that the radioactivity in caffeoylglycoside decreased rapidly between 5 and 20d. However, radioactive caffeic acid which might be released during the catabolism of caffeoyl glycoside was not detected. An attempt to trap free phenolic intermediates released during the catabolism of the hydroxycinnamoyl glycosides was carried out by feeding cold caffeic and ferulic acid. In this experiment, two attached 10d. old fruits were each fed three times with 30 μl aliquots of 0.76 μCi [$\text{U-}^{14}\text{C}$] cinnamic acid [$\text{SA } 460\text{mCi.mmol}^{-1}$] in 0,05M phosphate buffer at pH 7.0 with 12h. intervals between the applications. The fruits were then left to grow on the plant. A retardation of fruit growth was noted due to the application of this 90 μl solution of the precursor, despite the mode of application.

After 10d., one of the fruits, was injected with 50 μl of 0.05M phosphate buffer at pH 7.0 containing 25 μg of caffeic acid. The other fruit was fed with 50 μg of ferulic acid in the same volume of buffer. The amount of cold carrier added was kept to a minimum to avoid any toxic effect, but was still at a detectable level 6h. after feeding. It seems, visually fruit tissue can tolerate higher amounts of ferulate than caffeate. After 6h., the fruits were removed and the phenolics in each fruit extracted and fractionated according to the methods described in 2.3.2.1. Twenty microlitres of free phenolic extracts (total volume=1.0ml) were applied to a TLC-Silica gel, then developed with solvent system-I. The radioactive spots which correspond to

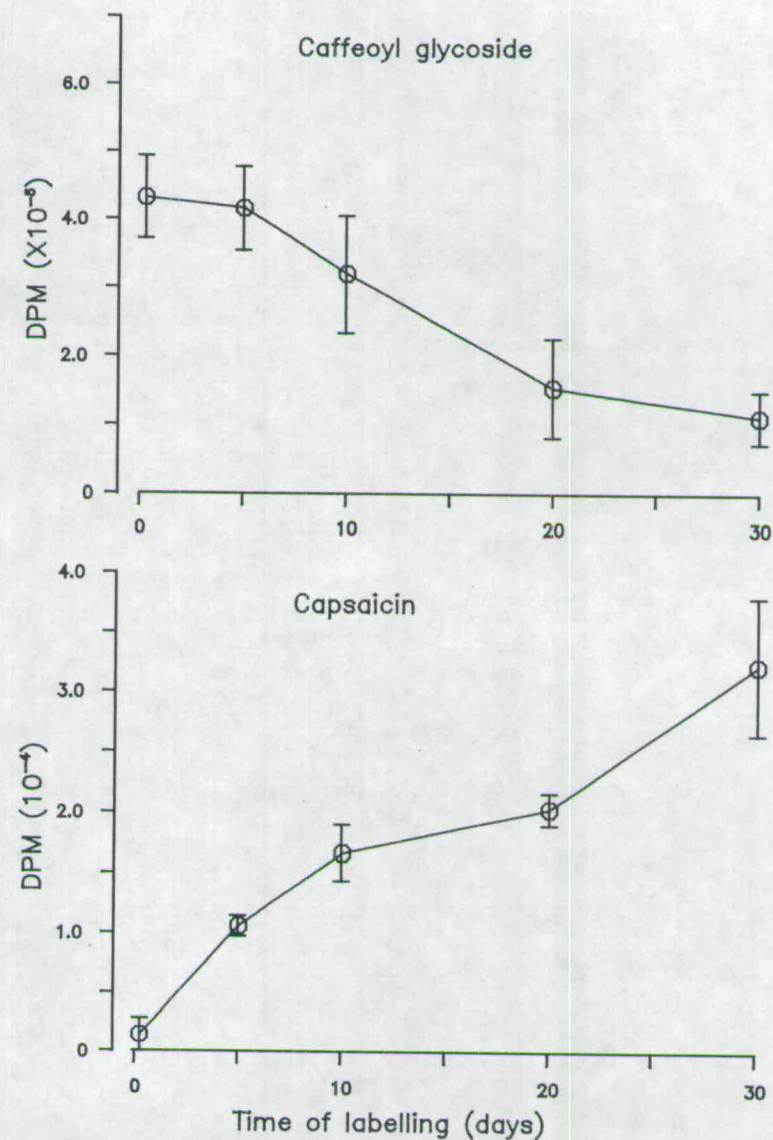
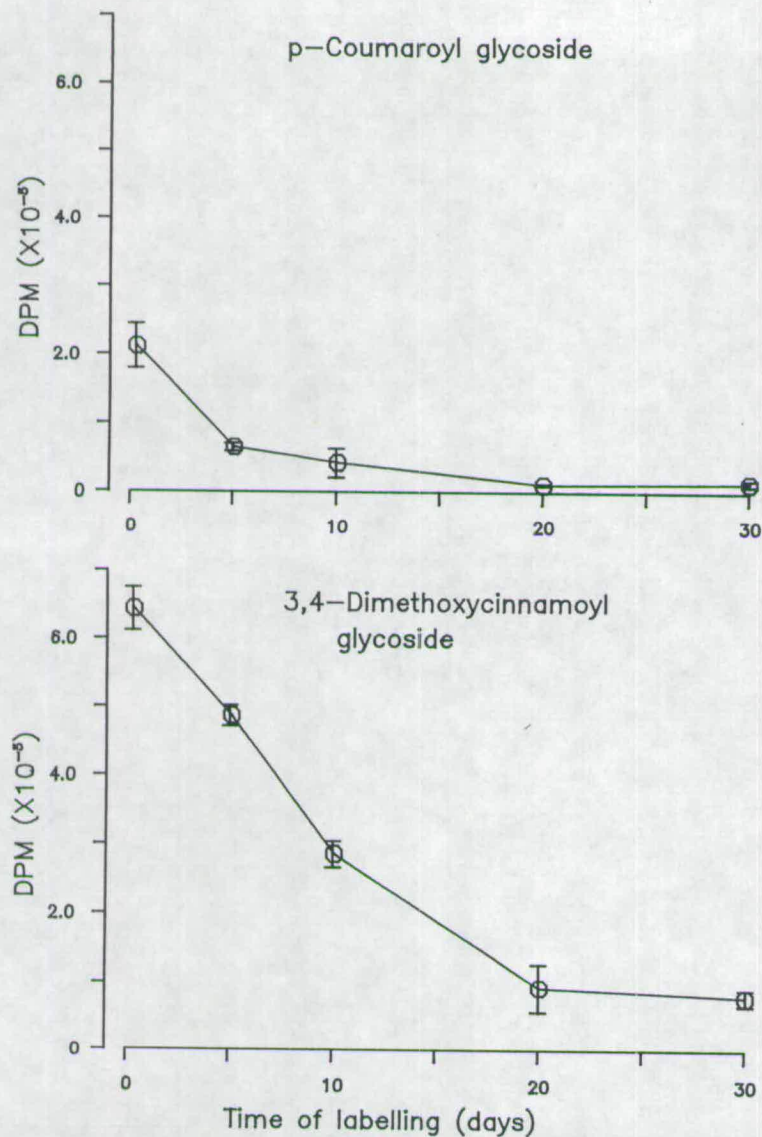


Fig. 3.6.3. Changes in the radioactivity in specific compounds during a long period of labelling in 10d. old fruits with $0.76\mu\text{Ci}$ $[\text{U-}^{14}\text{C}]$ cinnamic acid. Data are presented on *per* fruit basis with bars representing the SE of the mean of three replicates.

capsaicin, p-coumaric, caffeic and ferulic acid were scraped off and counted. Table 3.6.2 shows the radioactivity in the free phenolics released from their glycosylated form after trapping with caffeic and ferulic acid. The data presented are the actual counts (CPM).

Table 3.6.2. Distribution of [^{14}C] in free phenolics in 10d. old fruits labelled with $2.28\mu\text{Ci}$ [$\text{U-}^{14}\text{C}$] cinnamic acid for 10d. then fed with caffeic and ferulic acid for 6h.

Compound	+ Caffeate [CPM]	+ Ferulate [CPM]
Capsaicin	448	563
p-Coumaric acid	69	103
Caffeic acid	49	46
Ferulic acid	60	71

Radioactivity in the free phenolic intermediates, was very low in both trapping experiments. But these levels were still higher than background (25 ± 5 CPM). The higher than expected counts in p-coumarate were possibly due to contamination from capsaicin, since in the solvent system used both compounds are separated but their spots are close to each other.

3.6.4. β -Glucosidase activity in developing fruits

The activity of β -glucosidase in developing fruits was assayed in an attempt to relate the decrease in the level of the hydroxycinnamoyl glycosides with the change in the activity of the enzyme which catalyses the hydrolysis of these compounds. Ideally the experiment should be performed using a natural substrate. However, these substrates are difficult to obtain. In this experiment, a synthetic p-coumaroyl glucoside was used as substrate. $\sqrt{}$

At least 10 fruits at each of d.10, 15, 20, 25 and 30 were used as source of the enzyme. The methods used for extraction, protein determination and assay of the crude enzyme were all described in 2.6. Fig. 3.6.4. shows the change in activity of β -glucosidase during fruit development. As can be seen the activity of β -glucosidase

$\sqrt{}$ Therefore the data presented in Fig 3.6.4. could represent the activity of either p-coumaroyl- β -D-glucosidase or esterase.

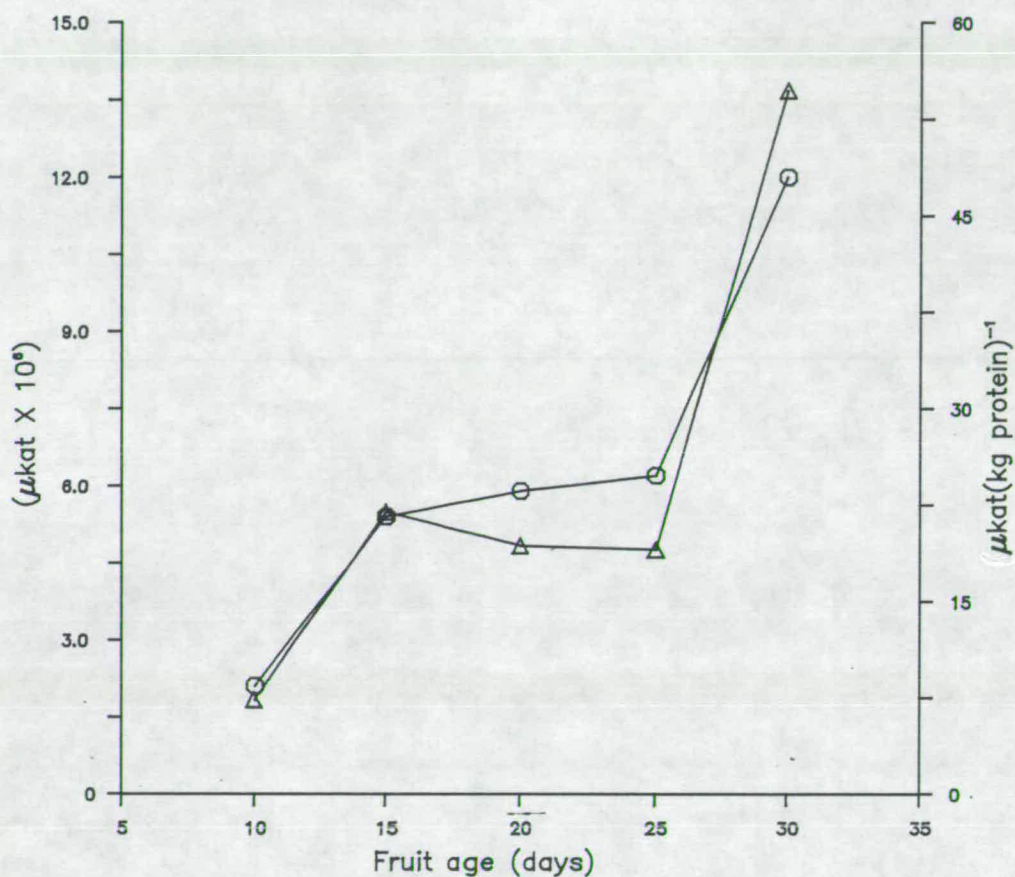


Fig. 3.6.4. β -Glucosidase in developing fruits. The activity was assayed using p-coumaroyl- β -D-glucose as substrate and according to the method described in 2.6. [O]— Activity in the assay mixture and [Δ]— specific activity.

was low in fruits of stage I (d.10) then increased and tended to plateau during the early and midpart of stage II (d.15-25). By the end of stage II the activity of β -glucosidase was high (d.25-30). A similar pattern of change can be seen if enzyme activity is expressed in units of specific activity. The change in the activity of β -glucosidase (p-coumaroyl- β -D-glucosidase) was consistent with the pattern of accumulation of hydroxycinnamoyl glycosides in the developing fruits.

3.6.5. Summary

The hydroxycinnamoyl glycosides synthesized and accumulated in fruits of stage I, were finally hydrolysed at a later stage and the products incorporated into lignin and capsaicin. There is some evidence to suggest that p-coumaric and caffeic acid are released from their glycosylated forms then rapidly incorporated into capsaicin and lignin. The hydrolysis product of 3,4-dimethoxycinnamoyl glycoside is probably incorporated into lignin only.

Chapter 4: DISCUSSION

4. DISCUSSION

A long term research aim of this laboratory is to develop cell cultures of *Capsicum frutescens* which consistently produce high levels of capsaicin. A variety of empirical approaches have already been employed in attempts to secure this aim, for example Yeoman *et al.* (1980, 1989), Lindsey and Yeoman (1984a,b), Lindsey (1985), but these have been only partially successful and it is now clear that further progress depends on acquiring a better understanding of the biosynthetic pathway leading to capsaicin. As most of this pathway is shared with other major metabolites (Yeoman *et al.*, 1980, Hall *et al.*, 1986), in particular those leading to the formation of the aromatic moiety (the phenylpropanoid pathway), it is important to study the relationship between phenylpropanoid metabolism and capsaicin synthesis. Previous research on the biosynthesis of capsaicin has concentrated on the conversion of the free phenolics, supposed intermediates in fruits and cell cultures (Hall *et al.*, 1986, 1987; Holden *et al.*, 1987a; Hall and Yeoman, 1991), while the water soluble conjugated phenolics have received little attention despite the fact that there is some evidence suggesting that some water soluble conjugated phenolics, such as glucose and SCoA esters of hydroxycinnamates, are active intermediates in phenylpropanoid metabolism (Brown, 1979; Molderez *et al.*, 1978, Moriguchi *et al.*, 1988 and the references therein; Zenk, 1979). In this thesis, the roles of the various groups of phenolics have been investigated in relation to capsaicin synthesis and accumulation, mainly in fruits but also in cultured cells.

This discussion is divided into five parts. The first three parts concentrate on aspects of phenylpropanoid metabolism before, during and after the onset of capsaicin synthesis in chilli pepper fruits. In the fourth part, an integrated analysis of the three preceeding parts is attempted in order to show how capsaicin synthesis may be regulated in developing fruits. The final part discusses how cell cultures metabolize phenylpropanoid compounds and how this is related to capsaicin synthesis in the plant.

4.1. Phenylpropanoid Metabolism in Fruits Before the Onset of Capsaicin Synthesis

4.1.1. Hydroxycinnamoyl glycosides are the main products of phenylpropanoid metabolism

The enzyme catalysing the conversion of phenylalanine to cinnamic acid, phenylalanine ammonia lyase (PAL) is, as expected, extremely active before the onset of capsaicin synthesis (Yeoman *et al.*, 1989; Holland, 1989). The product of this activity, cinnamate, leads into the phenylpropanoid pathway and provides intermediates leading to the aromatic moiety of capsaicin. At this early stage of fruit development, lignin is only accumulated to a very low level and the free phenolics, proposed as intermediates in capsaicin synthesis, were not detected by TLC and HPLC. This is consistent with the results of Hall *et al.*, (1987) and Holden *et al.*, (1987a) who presumed that these compounds are in a very small metabolic pool with a high rate of *turnover*. Perhaps significantly the most prominent products detected were two groups of water soluble conjugated phenolics. These were glycosylated flavonoids and glycosylated hydroxycinnamic acids. It is interesting to note that the balance between these two groups of compounds was affected by the mode of culture of the plants. Plants grown under glass (2.1) accumulated high levels of flavonoids while those in a controlled environment chamber (2.1) produced mainly hydroxycinnamoyl glycosides (see Table 4.1). These differences could be due to the different quality and quantity of light available in these growth conditions. Indeed it is well established, that the accumulation of flavonoids is greatly affected by light (Kreuzaler and Hahlbrock, 1973; Grisebach, 1979; Duell-Pfaff and Wellmann, 1982, Mohle *et al.*, 1985; Ibrahim, 1987).

It was also shown during tracer studies with [U-¹⁴C] cinnamic acid that the hydroxycinnamoyl glycosides were actively synthesized in fruits before the onset of capsaicin synthesis. Indeed the major part of the [U-¹⁴C] cinnamic acid added to fruits was incorporated into these substances with only a very small part in lignin and saponifiable cell wall phenolics. The remainder was incorporated into the other

groups of water soluble conjugated phenolics (Fig. 4.1). This is consistent with the high activity of PAL reported in fruits at this stage of development by Holland (1989) and Yeoman *et al.* (1989) leading directly to the provision of precursors for the synthesis and accumulation of the hydroxycinnamoyl glycosides.

Table 4.1. Two main groups of phenolics accumulated before the onset of capsaicin in fruits at the same stage of development grown in two different environments.

Compound	$\mu\text{mol per fruit}$	
	growth room (d.10)	glass house (d.20)
p-Coumaroyl glycoside	0.47	0.40
Caffeoyl glycoside	0.90	0.55
3,4-Dimethoxycinnamoyl glycoside	2.79	1.44
Flavonoids	0.55	3.11

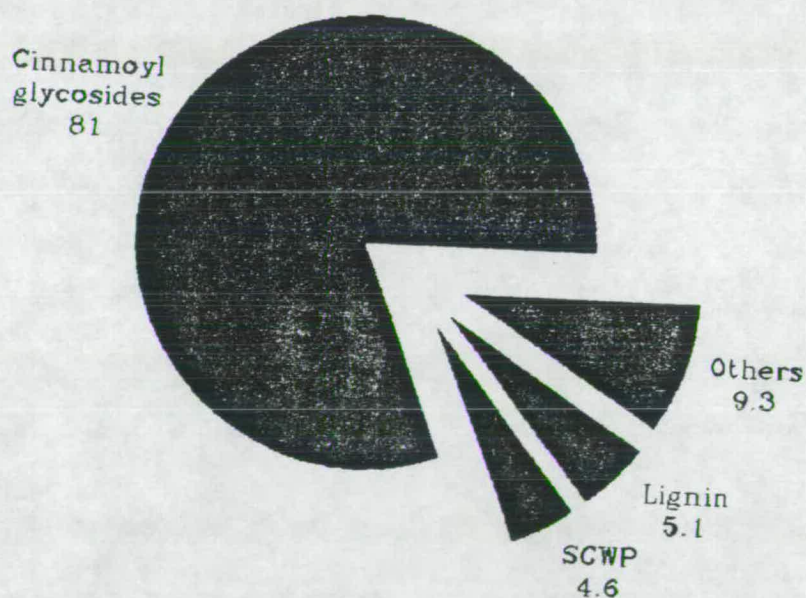


Fig. 4.1. Incorporation of [U-¹⁴C] cinnamic acid into different group of phenolics in fruits grown in a growth room before the onset of capsaicin synthesis (d.10). SCWP: Saponifiable cell wall phenolics, numbers= percentage of incorporation

4.1.2. Biosynthetic pathway leading to the hydroxycinnamoyl glycosides

Cinnamic, p-coumaric, caffeic, ferulic and sinapic acid are known to be the core metabolites in phenylpropanoid metabolism (Hanson and Havir, 1979). All of these molecules are subjected to condensation, esterification, reduction and side chain shortening to produce a variety of phenolic compounds in higher plants (Barz *et al.*, 1985). Investigations on the biosynthetic pathway leading to the hydroxycinnamoyl glycosides, which are the main products of phenylpropanoid metabolism before the onset of capsaicin synthesis will help to identify the branch point in phenylpropanoid metabolism leading to capsaicin and may help to show how the synthesis of capsaicin is regulated.

The role of p-coumaric acid, the hydroxylation product of cinnamic acid at the 4 position, as an intermediate in the biosynthesis of hydroxycinnamoyl glycosides, has been demonstrated (3.5), but there is still some uncertainty about the nature of the compounds between p-coumaric acid and the hydroxycinnamoyl glycosides. Stafford (1974) has suggested that the esterification of cinnamic acids is a terminal step. Caffeic and ferulic acid could be such intermediates, however, the extremely low level of radioactivity detected in these compounds after labelling with [U-¹⁴C] cinnamic acid and [U-¹⁴C] p-coumaric acid (3.5) suggests that these free acids are not intermediates in the synthesis of caffeoyl and 3,4-dimethoxycinnamoyl glycosides, although there is still a possibility that the pool sizes of these intermediates are very small and that glycosylation takes place rapidly. But it appears that the large differences between the radioactivity in p-coumaric acid and in caffeic or ferulic acid during pulse labelling with [U-¹⁴C] cinnamic acid is associated with how the hydroxycinnamoyl glycosides are synthesized from p-coumaric acid. An experiment in which [2-side chain-¹⁴C] caffeic acid was added to fruits in an attempt to eliminate the possibility that caffeic acid is an intermediate leading to caffeoyl glycoside was unsuccessful, due to the toxicity of caffeic acid (see 3.5.3).

An alternative is that caffeoyl and 3,4-dimethoxycinnamoyl glycoside are synthesized sequentially from p-coumaroyl glycoside. Caffeoylquinic acid

(chlorogenic acid), a typical ester commonly found in higher plants, appears to be synthesized from p-coumaroylquinic acid (Rhodes and Woollorton, 1976; Molderez *et al.*, 1978). It is also probable that 3,4-dimethoxycinnamoyl glycoside is synthesized from caffeoyl glycoside by methylation of both hydroxy phenolic groups. Methylation of flavonoids which are a product of a sequence of reactions between a hydroxycinnamic acid and three molecules of acetyl-S-CoA has also been suggested to take place at a late step, i.e. at the flavonoid stage rather than at the hydroxycinnamic acid stage (Grisebach, 1979; Hahlbrock, 1981). The results of the experiment with [U-¹⁴C] cinnamic acid in this study, although not convincing, do support the possibility that caffeoyl and 3,4-dimethoxycinnamoyl glycosides are synthesized sequentially via p-coumaroyl glycoside rather than from their corresponding free phenolics. Consistent with this is that the incorporation of [U-¹⁴C] cinnamic acid into p-coumaroyl and caffeoyl glycoside remained constant after reaching a maximum level, while incorporation into 3,4-dimethoxycinnamoyl glycoside kept increasing (see Fig. 3.5.3).

More convincing data were obtained from an experiment with [U-¹⁴C] p-coumaric acid, in which the period of labelling was 24h. instead of 6h. Here the incorporation of [U-¹⁴C] p-coumaric acid into p-coumaroyl and caffeoyl glycoside remained constant after 6h., although there was still a significant residual level of radioactivity in p-coumaric acid after 24h. The incorporation into 3,4-dimethoxycinnamoyl glycoside, however, was still increasing after 6h. (Fig. 3.5.6). A sequence of incorporation of [U-¹⁴C] p-coumaric acid into these three glycosides was also observed. Caffeoyl and 3,4-dimethoxycinnamoyl glycoside were not significantly labelled at 5min., but by this time p-coumaroyl glycoside was already labelled.

If p-coumaroyl glycoside is intermediate to caffeoyl glycoside, the incorporation of [U-¹⁴C] cinnamic acid into caffeoyl glycoside should be slow due to the dilution of newly formed radioactive intermediate with a substantial amount of non-radioactive endogenous intermediate. But this was not the case, as the incorporation of [U-¹⁴C] cinnamic acid into caffeoyl glycoside was as rapid as into p-coumaroyl

glycoside (see Fig. 3.5.3). One possible explanation for this pattern of results is as follows. There may be two pools of hydroxycinnamoyl glycosides, an active metabolic pool and a storage pool. Assuming the size of the metabolic pool is very small, once p-coumaroyl glycoside is formed, it will in part be rapidly converted to caffeoyl glycoside and the other part translocated into the storage pool in which the level of intermediate will be substantial. It is proposed that the same thing happens with caffeoyl glycoside. It has been suggested that the storage pool is in the vacuole (Pridham, 1965; Hrazdina and Wagner, 1985, Renaudin and Guern 1990) and this pool is metabolically inactive (Wagner, 1981; Wagner and Matile, 1985; Hrazdina and Wagner, 1985). Under these circumstances, no significant decrease in radioactivity in p-coumaroyl and caffeoyl glycoside after, reaching a maximum level, would be expected in the experiment with [U-¹⁴C] cinnamic acid and [U-¹⁴C] p-coumaric acid. The slow decrease in radioactivity in p-coumaroyl and caffeoyl glycoside at the end of the experiment could be due to the involvement of a reversible translocation from the storage pool to the metabolic pool. The scheme presented in Fig. 4.2. shows the proposed interconversion between the three hydroxycinnamoyl glycosides.

Clearly, further work is required to prove this proposed pathway. Pulse chase labelling of the hydroxycinnamoyl glycosides in fruits at this early stage with high SA [U-¹⁴C] cinnamic acid in which the tracer is rapidly incorporated mainly into these substances would be useful to test this possibility. Enzymatic evidence is also required to support the proposed pathway. However, before the enzymatic experiments can be conducted, the detailed structure of the three glycosides will need to be elucidated.

4.1.3. The possible role of p-coumaroyl-SCoA in the biosynthesis of hydroxycinnamoyl glycosides

It has been suggested that p-coumaroyl-SCoA (see 3.4 and 3.5) is present in fruits before the onset of capsaicin synthesis. There are four reasons for suspecting that Fr-9 is p-coumaroyl-SCoA. Firstly, it was present as a water soluble conjugated phenolic.

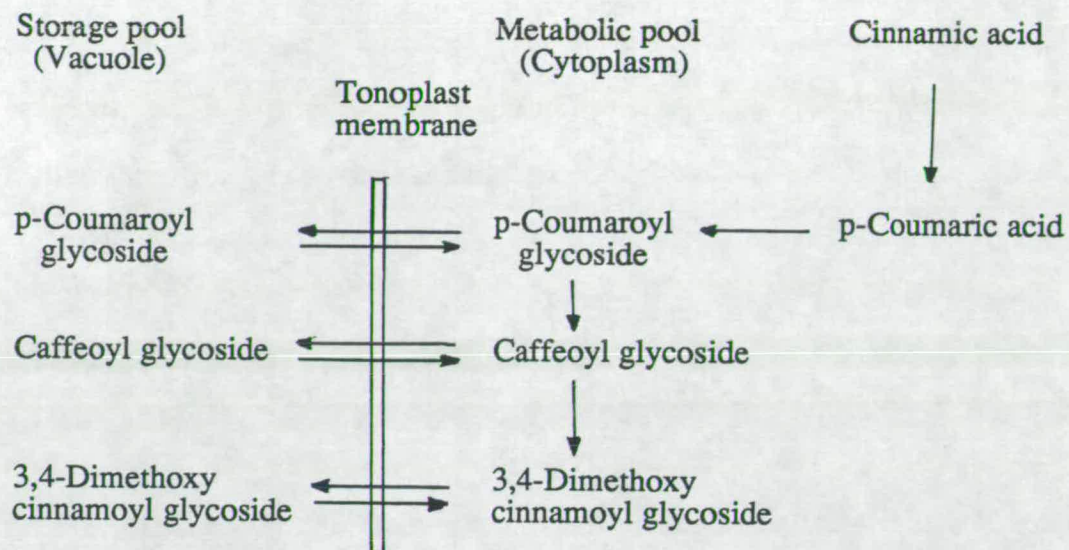


Fig. 4.2. Proposed scheme for the interconversion and translocation between cellular compartments of hydroxycinnamoyl glycosides in cells of fruits of *Capsicum frutescens*

Secondly, it appeared to be an active intermediate as shown by its rapid synthesis from p-coumaric acid and subsequent metabolism. Thirdly, Fr-9 was not detectable by HPLC which is consistent with it being an active intermediate in a small pool with a rapid turnover. Fourthly, it was the first labelled substance detected following labelling with radioactive p-coumaric acid. Fr-9 was the only substance labelled after a one minute pulse with [3,5- ^3H]-p-coumaric acid (data not presented in this thesis). These results suggest that Fr-9 is very close to p-coumaric acid.

It has been well established that p-coumaroyl-SCoA is involved in the synthesis of flavonoids, lignin (Hahlbrock, 1977, 1981; Grisebach, 1979, 1981; Gross, 1979; Zenk, 1979) and esters of hydroxycinnamic acids (Gross, 1981). Therefore, it is not surprising that Fr-9 is p-coumaroyl-SCoA and is present in fruits during the active synthesis of capsaicin, because at this stage, the fruits are also actively synthesizing lignin. It has already been established that the main products of phenylpropanoid metabolism in fruits of this early stage are the hydroxycinnamoyl glycosides, with only small contribution to the synthesis and accumulation of flavonoids, lignin and saponifiable cell wall phenolics. It has also been suggested that p-coumaroyl-SCoA

may be involved in the biosynthesis of these hydroxycinnamoyl glycosides, probably as an active intermediate between p-coumaric acid and p-coumaroyl glycoside. On the otherhand, several reports have suggested that glycosylation is catalysed by a glycosyl transferase via a transglycosylation between a UDP-sugar and an aglycone (Hosel, 1981; Villegas and Kojima, 1986). Clearly, further work is required to distinguish between these possibilities. In this study it has proved difficult to confirm the presence of p-coumaroyl-SCoA in fruits, because if it is Fr-9, it is present only at a very low level and is not highly radioactive. An attempt at simple identification by comparing the R_t value of Fr-9 with a synthetic p-coumaroyl-SCoA is necessary.

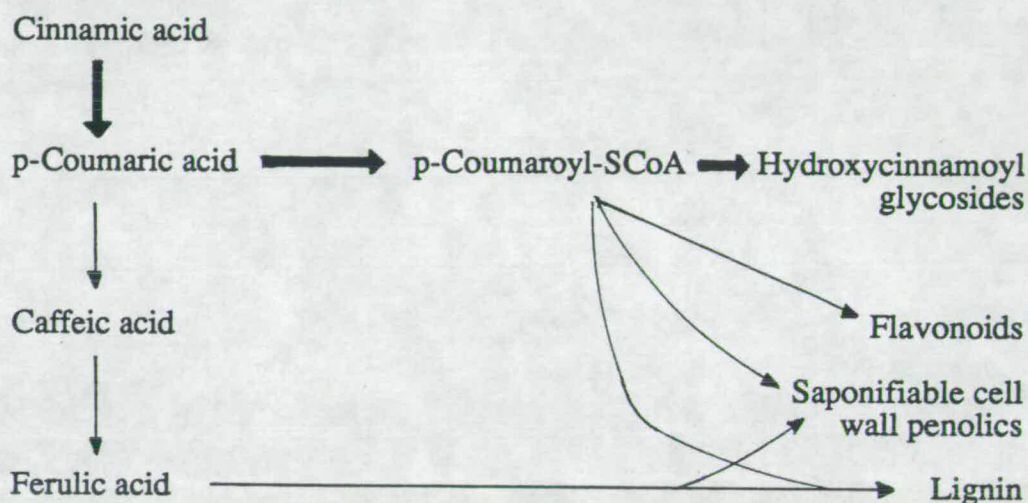


Fig. 4.3. Proposed phenylpropanoid metabolism in *Capsicum frutescens* before the onset of capsaicin synthesis. The thick line indicates that most of the p-coumaric acid is diverted into hydroxycinnamoyl glycosides.

A demonstration of the presence p-coumaroyl-SCoA ligase activity in the placenta and seed in which flavonoids are not synthesized and the synthesis of lignin is not yet active before the onset of capsaicin synthesis, would support the possibility that p-coumaroyl-SCoA is involved in the biosynthesis of the hydroxycinnamoyl glycosides in chilli pepper fruits. So far the involvement of hydroxycinnamoyl-SCoA in the biosynthesis of hydroxycinnamoyl sugar esters has not been investigated. A simplified outline presented in Fig. 4.3. summarizes some of the possible events of phenylpropanoid metabolism in fruits before the onset of capsaicin synthesis.

4.2. Phenylpropanoid Metabolism During The Onset of Capsaicin Synthesis

4.2.1. Products of phenylpropanoid metabolism

It has already been shown in this thesis that during the onset of capsaicin synthesis, fruits also actively synthesize lignin and the C₆-C₁ groups of phenolics such as vanillic acid glycoside, p-glycosidobenzaldehyde and some other unknown phenolics. It is as expected that C₆-C₁ groups of phenolics are synthesized actively at the same time as capsaicin during fruit development because in higher plants they are all derived from C₆-C₃ (phenylpropanoid) groups of compounds via β -oxidation. In fruits at this stage, hydroxycinnamoyl glycosides were also synthesized, but their rate of synthesis was much lower than before the onset of capsaicin synthesis.

It has been suggested by Hall *et al.* (1987), Holden *et al.* (1987a), and Hall and Yeoman (1991) that the saponifiable cell wall phenolics are a major sink of phenylpropanoid metabolism in cell cultures of *Capsicum frutescens*. This is surprising because these groups of phenolics do not appear to be a major product of phenylpropanoid metabolism in chilli pepper fruits. Indeed, after feeding [U-¹⁴C] cinnamic acid, only 10 to 12% of the [¹⁴C] was incorporated into these compounds. If the saponifiable cell wall phenolics are further fractionated according to the methods routinely used during the investigation, only 1% of the radioactivity was found in the free phenolics. This shows that only a very small part of the free phenolics are bound via an ester link with cell wall polymer. Possibly most of the phenolic moieties of the saponifiable cell wall phenolics are still bound to sugars via an O-glycosidic link which is stable under the conditions used for saponification. Indeed, a large proportion of saponifiable cell wall phenolics of spinach cell cultures are also present as alkali stable conjugates as shown by their immobility on TLC with C₆-H₆-HOAc (9:1) as solvent system (Fry, 1984).

The presence of p-coumaric, caffeic and ferulic acid, and free phenolics which may be involved in the biosynthesis of lignin was also demonstrated by feeding with

[U-¹⁴C] cinnamic acid. Even during the experiment with [U-¹⁴C] p-coumaric acid which was taken up slowly by the cells from the locus, the presence of a free phenolic tentatively identified as p-coumaraldehyde, a precursor immediately after p-coumaric acid in lignin biosynthesis, was observed. This shows the possibility of an increase in the pool sizes of proposed free phenolic intermediate in lignin and capsaicin biosynthesis during fruit development. Changes in the flux of the active intermediates may also take place which leads to an active synthesis of capsaicin and lignin.

4.2.2. The intermediates of capsaicin biosynthesis

Without the evidence from experiments with radioactive precursors, it would appear that some of the water soluble conjugated phenolics may be intermediates in capsaicin biosynthesis, because the decrease in the amounts of these compounds during fruit development coincides with an increase in the amount of capsaicin (see 3.2). However, using tracers, it has been demonstrated that free phenolics are the most likely candidates as intermediates in capsaicin synthesis. There is a body of evidence to support this suggestion. 1. p-Coumaric, caffeic and ferulic acid are all active intermediates, as shown by their rapid synthesis from cinnamic acid followed by their rapid metabolism (see 3.4.1). 2. Increases in the pool sizes of free phenolic intermediates were observed during fruit development (see 3.3). But, it should be pointed out that the level of these intermediates is extremely small and not measurable without the use of radioactive tracers. 3. The disappearance of radioactivity in free phenolics ^{after 60 min} coincided with the increase in incorporation of precursor into capsaicin e.g. after feeding with [U-¹⁴C] cinnamic acid (see 3.4.1). 4. The incorporation of [U-¹⁴C] cinnamic acid and [U-¹⁴C] p-coumaric acid into capsaicin was very rapid which is consistent with small pool sizes and a rapid turnover of the intermediates.

The results reported in this thesis confirm the proposed pathway of capsaicin biosynthesis reported by Yeoman *et al.* (1980, 1989), Kopp *et al.* (1983), Hall *et al.* (1986, 1987) and Holden *et al.* (1987a). Vanillin and vanillylamine, however,

were not detected in fruits during experiments with [U-¹⁴C] cinnamic acid. This could be due to the distance of vanillin and vanillylamine from the precursor, or that the pool sizes of these compounds which are intermediates specifically for the synthesis of capsaicin are much smaller than those for p-coumaric, caffeic and ferulic acid (general intermediates in phenylpropanoid metabolism). So far there have been no tracer studies on capsaicin biosynthesis with radiolabelled vanillin to confirm its role as an intermediate in capsaicin biosynthesis. On the other hand, the role of vanillylamine in capsaicin biosynthesis has been clearly demonstrated by tracer studies (Bennet and Kirby, 1968; Holland, 1989) and enzymatically (Iwai *et al.*, 1977; Holland, 1989).

4.2.3. Why p-coumaric acid is poorly incorporated into capsaicin?

According to the proposed biosynthetic pathway for capsaicin, p-coumaric acid is closer to capsaicin than cinnamic acid (Fig. 4.5). Therefore, in studies using [U-¹⁴C] p-coumaric acid, it was expected that the capsaicin intermediates after p-coumaric acid would be revealed. However, the incorporation of [U-¹⁴C] p-coumaric acid into the capsaicin intermediates further down the pathway than p-coumaric acid was much lower than with [U-¹⁴C] cinnamic acid. Apart from lignin and saponifiable cell wall phenolics, the incorporation [U-¹⁴C] p-coumaric acid into capsaicin and the regular products of phenylpropanoid metabolism was also much lower than with [U-¹⁴C] cinnamic acid (see Table 4.3).

Poor incorporation of [U-¹⁴C] p-coumaric acid into active intermediates could be due to the slow uptake of precursor from the locus, but this should not affect the eventual level of incorporation into the end product of metabolism (capsaicin). Indeed, the incorporation of [U-¹⁴C] p-coumaric acid into capsaicin was even lower than with [U-¹⁴C] phenylalanine [Bladon, 1991 (personal communication)]. This pattern of results could be explained by the formation of an irregular metabolite (Fr-6) which was always present in experiments with high SA radiolabelled p-coumaric acid.

The idea that Fr-6 is an irregular metabolite is based on the fact that this

compound was not detected by HPLC, and appeared not to be further metabolized. p-Coumaric acid synthesized from cinnamic acid *in vivo* was not incorporated into this compound and the pattern of synthesis was not affected by fruit development. The danger of the formation of irregular metabolites from supplied precursors has been pointed out by Barz & Hosel (1979) and Barz and Koster (1981).

Table 4.3.3. Comparison in the radioactivity of various phenolics in 20d. old fruits fed with 0.76 μ Ci [U-¹⁴C] cinnamic acid for 6h. and 1.2 μ Ci [U-¹⁴C] p-coumaric acid for 24h.

Compounds	Activity [DPM]	
	Cinnamic acid	p-Coumaric acid
Cinnamic acid	24,800	0
p-Coumaric acid	-	28,000
p-Coumaroyl glycoside	45,600	50,500
Caffeoyl glycoside	62,000	16,650
3,4-Dimethoxycinnamoyl glycoside	130,800	24,000
Saponifiable cell wall phenolics	214,500	260,165
Lignin	525,470	565,393
Fr-6	-	464,900
Capsaicin	32,500	10,080

Note: Data are presented as an average of three replicates on a *per* fruit basis.

The chemical nature of Fr-6 may be deduced from the results of tracer studies and its behaviour in the liquid-liquid extraction procedure used routinely. Both [3,5-³H] and [U-¹⁴C] p-coumaric acid were extensively incorporated into Fr-6.

Its presence in the aqueous fraction during liquid-liquid extraction (2.3.2.1) suggest that the compound is a water soluble conjugated phenolic and possibly an O- or ester glycoside of

p-coumaric acid. Indeed the R_t value of Fr-6 is the same as that of synthesized p-coumaroyl- β -D-glucoside. Because this compound does not appear to be metabolized while p-coumaroyl- β -D-glucose is a good substrate for a crude β -glucosidase extracted from fruits of this age (d.20), suggest that the compound is an O-glycoside of p-coumaric acid. Further simple tests using alkaline, acid and enzyme hydrolysis should reveal the nature of this conjugate. The ability of plant cells to glycosylate exogenous phenolics has been reported frequently (Furuya, 1978; Furuya *et al.*, 1987; Tabata *et al.* 1988; Kodama *et al*, 1990; Kokubo *et al*, 1991a&b).

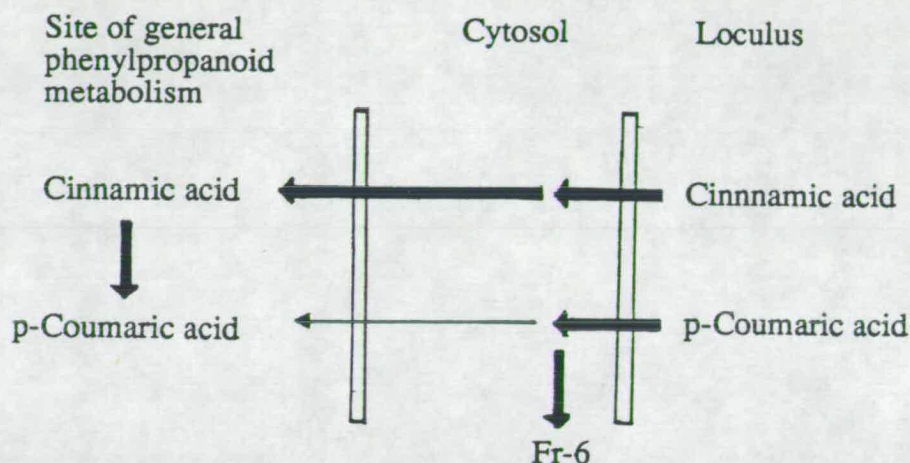


Fig. 4.4. Formation of Fr-6 from p-coumaric acid during labelling with a high SA [3,5-³H] and [U-¹⁴C]-p-coumaric acid

The scheme presented in Fig. 4.4. proposes a simplified mechanism to explain why p-coumaric acid is incorporated less efficiently into capsaicin. There is no doubt that a plant cell is much more complicated than a reaction tube because of the elaborate compartmentation within the cell and this compartmentation has an important role in the regulation of metabolism (Oaks and Bidwell, 1970). It would seem that the conversion of p-coumaric acid into Fr-6 takes place before p-coumaric acid has reached the site of central phenylpropanoid metabolism, because endogenous p-coumaric acid formed from cinnamic acid is not converted into Fr-6.

4.2.4. The role of hydroxycinnamoyl glycosides and flavonoids in capsaicin biosynthesis

It has been demonstrated in this thesis that hydroxycinnamoyl glycosides are

intermediates in phenylpropanoid metabolism in chilli pepper fruits. These compounds also act as intermediates in capsaicin biosynthesis, but they are more likely to be storage intermediates rather than direct intermediates. There is some evidence to support this suggestion. Firstly, these compounds were produced actively before the onset of capsaicin synthesis. Secondly, the decrease in radioactivity in these compounds was slow after reaching maximum levels in experiments with [U-¹⁴C] cinnamic acid and [U-¹⁴C] p-coumaric acid (3.4.1 and 3.4.3), while the incorporation of these precursors into capsaicin and lignin, which are synthesized at the same stage of development in fruits, was rapid. In addition, the role of phenylpropanoid glycosides as storage intermediates in lignin biosynthesis has also been reported by Marcinowski and Grisebach (1977).

So far, there is no strong evidence to suggest which glycosides act as precursors in capsaicin biosynthesis. p-Coumaroyl and caffeoyl glycosides are prime candidates because their free phenolics are active intermediates in capsaicin biosynthesis. The possibility that 3,4-dimethoxycinnamoyl glycoside may act as a precursor in capsaicin biosynthesis, however, cannot be excluded. Demethylation of 3,4-dimethoxycinnamic acid after release from the glycoside may take place in fruits, the product then being incorporated into capsaicin and lignin. Indeed, it has been recently suggested that vanillic acid is synthesized from cinnamic acid via 3,4-dimethoxycinnamic acid (Brodelius and Funk, 1990; Funk and Brodelius, 1990a,b). Demethylation of methoxycinnamic acids has also been reported by Pridham (1965). In addition, demethylation also occurs during the biosynthesis of morphine from thebaine (Swain, 1965; Brochmann-Hanssen, 1985)

An attempt to discover how the hydroxycinnamoyl glycosides are involved in capsaicin biosynthesis has also been made in this thesis (3.6). The evidence obtained suggests that free hydroxycinnamic acids may be released from their glycosides, and subsequently act as active intermediates in phenylpropanoid metabolism. A high activity of β -glucosidase (p-coumaroyl- β -D-glucosidase) in fruits during the onset of capsaicin synthesis also supports this suggestion. The release of free phenolics from a bound form before being further metabolized has also been proposed by Barz and

Koster (1981) and the hydrolysis of these glycosides may take place outside the vacuole (Marcinowski and Grisebach, 1978; Marcinowski *et al.*, 1979, Burmeister and Hosel, 1981, Barz and Koster, 1981).

The role of flavonoids in capsaicin biosynthesis has not been studied in detail in this thesis, because these compounds are not major components in the fruits used during the investigation. In addition, these compounds are mainly accumulated in the pericarp, which is not the site of capsaicin synthesis and accumulation. However, it is interesting to note the pattern of accumulation of these compounds during fruit development in which they are accumulated actively in the early stage and subsequently disappear during the onset of capsaicin synthesis (3.2). Are these compounds synthesized in actively elongating fruits to protect the tissues beneath the epidermis [the main site of accumulation for flavonoids (Wiermann, 1981)] as suggested by Hahlbrock (1981), Hahlbrock and Scheel (1989), Chappell and Hahlbrock (1984) and Ohl *et al.* (1989)? Do they also take part in the synthesis of capsaicin and lignin? It has been reported by Barz & Hosel (1979) and Barz & Koster (1981) that during degradation, flavonoids release hydroxycinnamates and hydroxybenzoates. In the case of flavonoids in pepper fruits all flavonoids are hydroxylated at the 3' and 4' positions, therefore caffeic acid could be released from these flavonoids and subsequently transported from the pericarp into the placental and seed tissues acting as precursors for capsaicin and lignin biosynthesis. Clearly, tracer studies are required to test the involvement of flavonoids in capsaicin biosynthesis.

The scheme presented in Fig. 4.5. tentatively summarizes the metabolism of phenylpropanoids during the onset of capsaicin synthesis. It is visualised that two major sources provide the intermediates for capsaicin and lignin biosynthesis, these are the hydroxycinnamoyl glycosides and free phenolics directly synthesized from cinnamic acid.

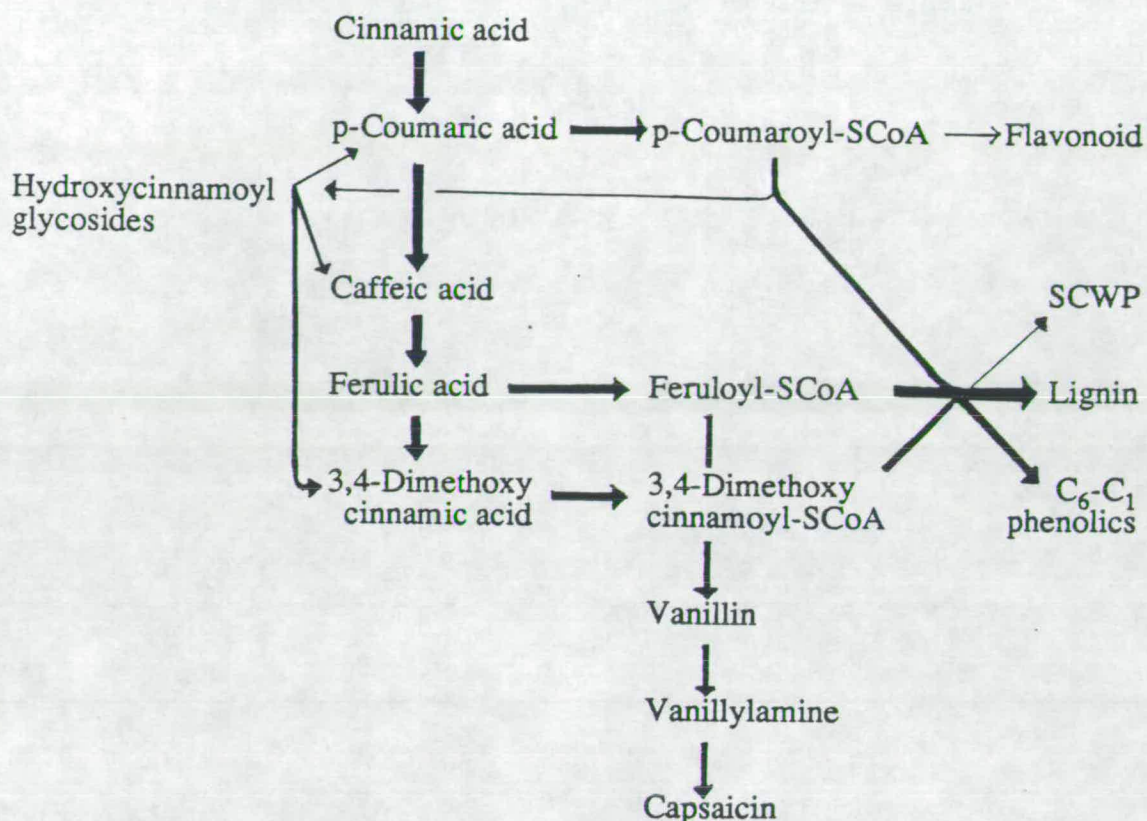


Fig. 4.5. Proposed phenylpropanoid metabolism in fruits of *Capsicum frutescens* during the onset of capsaicin synthesis. SCWP: Saponifiable cell wall phenolics

4.3. Phenylpropanoid metabolism after the onset of capsaicin synthesis

Phenylpropanoid metabolism in fruits after the onset of capsaicin synthesis was not studied in detail in this thesis. Some important points, however, can be drawn from the results presented in 3.2 and 3.3. [^{14}C] Cinnamic acid added to attached fruits at this stage (d.40), was metabolized rapidly. There was a high incorporation of [^{14}C] into the C₆-C₁ groups of phenolics such as the vanillic glycosides and some unknown glycosides. Some of the compounds not detected by HPLC but which were highly labelled may act as intermediates during the conversion of C₆-C₃ into C₆-C₁ groups of phenolics.

The results presented in Fig. 3.2.9. show that certain C₆-C₁ phenolic glycosides such as the glycoside of vanillic acid and p-hydroxybenzaldehyde appeared to be

further metabolized. This metabolism coincides with fruit ripening. It was suggested that hydroxybenzoic acids were degraded by peroxidase to produce quinone (Barz and Hosel, 1979). In pepper fruits, the degradation product may be the water soluble conjugated phenolics which were not retained during HPLC analysis (peak-5, see Fig. 3.2.2). The reaction scheme presented in Fig. 4.6 shows the proposed phenylpropanoid metabolism in chilli pepper fruits after the onset of capsaicin synthesis.

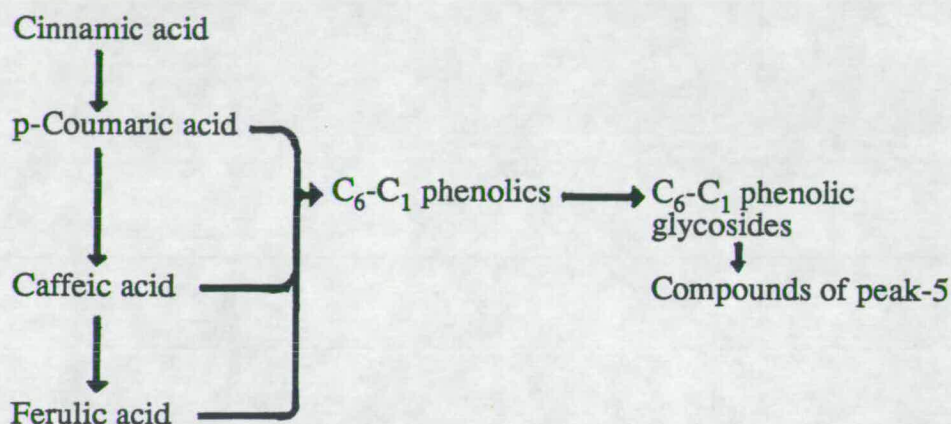


Fig. 4.6. Proposed phenylpropanoid metabolism in fruits of *Capsicum frutescens* after the onset of capsaicin biosynthesis.

4.4. Regulation of Capsaicin Biosynthesis

There are three major factors involved in metabolism, these are compartment, substrate and the enzyme. Reactions will not take place in the absence of one of these factors. If the substrate and enzyme are available, for example, but are located in different compartments, the reaction will not proceed. In capsaicin biosynthesis, there are two independent pathways, one producing the aromatic moiety of capsaicin, the other the fatty acid chain (see Fig. 1). It has been suggested by Lindsey (1986a) that the rate limiting step in capsaicin synthesis in cell cultures is the aromatic branch of the pathway. However, this may well depend on the cell line being used. Indeed, it has been shown by Yeoman *et al.* (1980) and Lindsey and Yeoman (1984a,b) that capsaicin yield can be dramatically increased in cell cultures by the addition of

isocaproic acid, an intermediate to capsaicin in the fatty acid branch. In fruits of *Capsicum frutescens*, capsaicin synthase is present before the onset of capsaicin synthesis (Holland, 1989, Yeoman *et al.*, 1989) and therefore, they have suggested that before the onset of capsaicin synthesis, capsaicin synthase is subjected to substrate limitation.

The available evidence from previous investigations (Holland, 1989 and Yeoman *et al.*, 1989) and from this study, show that the reaction steps in the pathway between phenylalanine and p-coumaric acid are operating actively before the onset of capsaicin synthesis. While these reaction steps may control general phenylpropanoid metabolism, they are not considered to be the limiting steps in capsaicin biosynthesis (Yeoman *et al.*, 1989; Hall and Yeoman, 1991). Tracer studies carried out with fruits before the onset of capsaicin synthesis using [U-¹⁴C] cinnamic acid and [U-¹⁴C] p-coumaric acid showed poor incorporation of these precursors into caffeic and ferulic acid. This contrasts sharply with the high levels of radioactivity found in caffeic and ferulic acid after feeding with [U-¹⁴C] cinnamic acid during the onset of capsaicin synthesis. This low level of radioactivity in caffeic and ferulic acid was also consistent with low incorporation of [U-¹⁴C] cinnamic acid into lignin. Under these conditions, capsaicin, was of course not labelled. Therefore, the reaction steps between p-coumaric and ferulic acid may not control the biosynthesis of capsaicin alone, but also the synthesis of lignin. It would seem, there must be another reaction step which is a prerequisite for the synthesis of capsaicin to proceed which may be one of the reactions between ferulic acid and vanillylamine in the latter part of the pathway leading to capsaicin.

How then may vanillylamine be formed from the hydroxycinnamic acids. There are a number of possibilities which have not been fully tested. One possibility proposed by Yeoman *et al.* (1980) suggested that vanillin is synthesized from ^{caffeic acid via} protocatechuic aldehyde. Another is that vanillin is formed from p-coumaric acid via p-hydroxybenzaldehyde. This aldehyde could then undergo subsequent hydroxylation and methylation at the 3 position to yield vanillin. Transamination to produce vanillylamine requires a carbonyl group, but this does not have to be from vanillin.

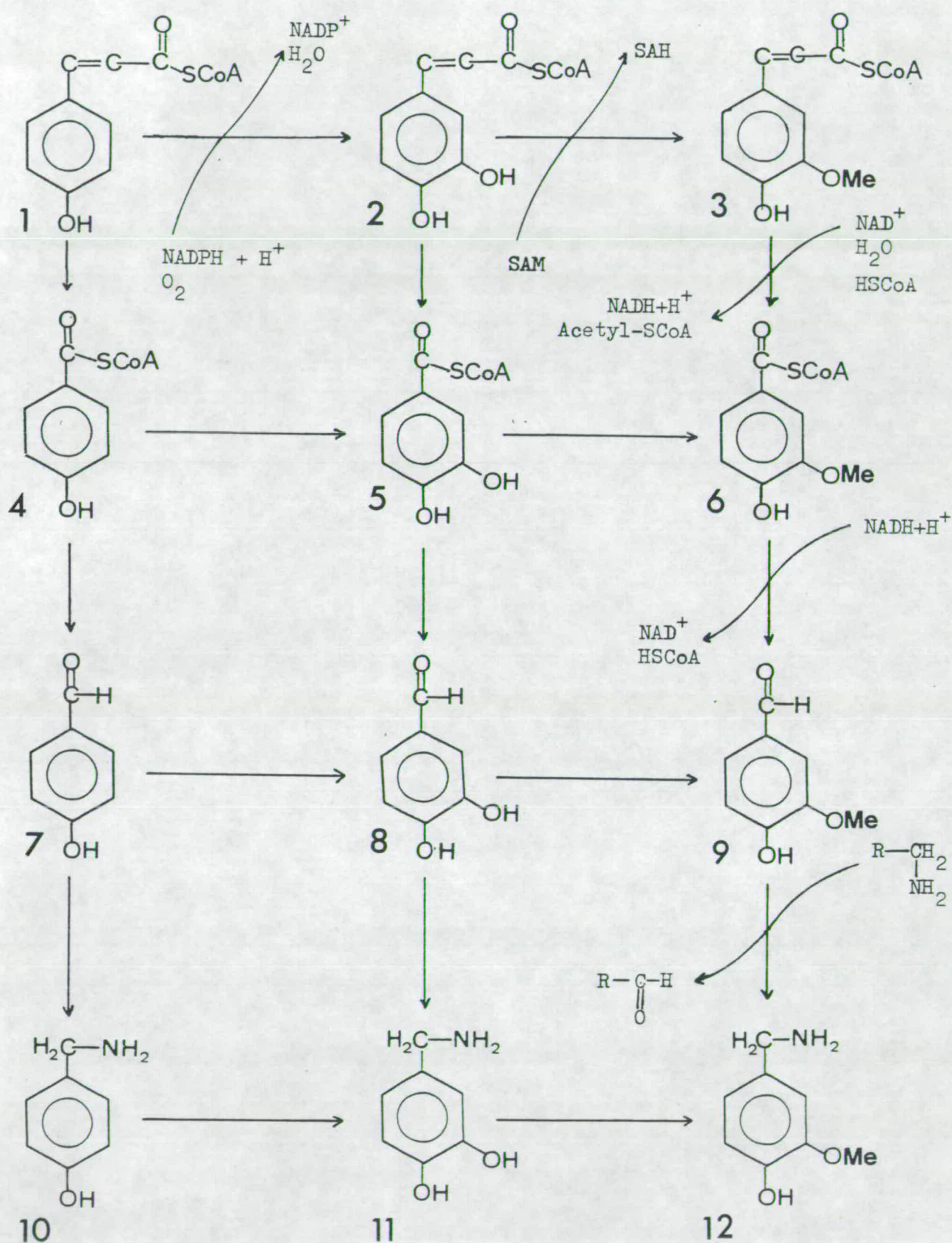
The transamination could also take place with either p-hydroxybenzaldehyde or protocatechuic aldehyde with hydroxylation or methylation taking place after this reaction. In such a pathway, vanillin would not be an intermediate in capsaicin biosynthesis which is consistent with the inability in this study to detect radioactive vanillin in fruits after a short pulse labelling with [U-¹⁴C] cinnamic acid during the onset of capsaicin synthesis. Indeed, at this stage of development (15d.) two unknown radioactive spots with R_f values close to caffeic acid indicating similar patterns of hydroxylation in the aromatic ring appeared to be active intermediates in phenylpropanoid metabolism. Fig. 4.7 shows a scheme of the various possible pathways leading to vanillyamine. Although all of these pathways are possible, in chilli pepper fruits, there may be only a single pathway leading to vanillyamine. Before this pathway is established it would be difficult to decide which reaction is the limiting step in capsaicin biosynthesis.

4.5. Phenylpropanoid Metabolism in Cell Cultures

Pepper cell cultures have the ability to produce a variety of phenolic compounds such as the glycosides of cinnamates and benzoates, and lignin (3.1). The activities of the first two enzymes in the pathway from phenylalanine leading to capsaicin (PAL and cinnamate 4-hydroxylase) have also been reported by Holland (1989) to be present in cell cultures. Fluctuations in the levels of some phenolic glycosides during the culture period have been observed, but capsaicin, one of the end products of phenylpropanoid metabolism in chilli pepper fruits, was not detected in the cultures used in this investigation. Success in increasing capsaicin production in cultures, on the otherhand, has been achieved by the addition of precursors such as phenylalanine (Yeoman *et al.*, 1980; Lindsey and Yeoman, 1984b), ferulic acid (Lindsey, 1986a; Johnson *et al.*, 1990), isocaproic acid (Yeoman *et al.*, 1980; Lindsey and Yeoman, 1984a,b); cell immobilization (Lindsey, 1985, 1986ab; Lindsey and Yeoman, 1983, 1984a,b; Lindsey *et al.*, 1983; Ravishankar *et al.*, 1988; Johnson *et al.*, 1990); nitrate limitation (Lindsey, 1985, 1986b) and using PFP resistant (phenylalanine over producer) cultures (Salgado-Graciglia and Ochoa-Alejo, 1990). These results presumably indicate the differences in the cell lines used by these workers in which

Fig. 4.7

Possible pathways leading to the formation of vanillylamine from hydroxycinnamic acids (other cofactors may also be involved in these reactions). 1. p-Coumaroyl-SCoA, 2. Caffeoyl-SCoA, 3. feruloyl-SCoA, 4. p-hydroxybenzoyl-SCoA, 5. proto catechuoyl-SCoA, 6. vanilloyl-SCoA, 7. p-hydroxybenzaldehyde, 8. Protocatechuic aldehyde, 9. vanillin, 10. p-hydroxybenzylamine, 11. protocatechuylamine, 12. vanillylamine.



all reactions in the pathway leading to capsaicin are operating. Indeed, capsaicin is only accumulated in certain cell lines (Holden *et al.*, 1986; 1987a).

Perhaps, the extremely low activities of the enzymes involved in the steps leading to capsaicin are responsible for poor capsaicin synthesis in cultures used in this investigation. Increasing PAL activity by elicitation (Holden *et al.*, 1988a,b) did increase the flux of intermediates in the pathway leading to capsaicin synthesis, but in the cell cultures used, capsaicin was not detected by HPLC (Holden, 1988b). In addition, the synthesis of lignin has also been blocked by supplying sinapic acid to immobilized cell cultures which it was anticipated would reduce the flux of ferulic acid into lignin, but here again the synthesis of capsaicin did not increase. All of these observations are consistent with the suggestion made earlier (4.3) that the limiting reaction in capsaicin biosynthesis is located at the end of the pathway, probably between the β -oxidation product of hydroxycinnamic acid (possibly hydroxybenzoyl-SCoA) and capsaicin. This view is supported by the ability of cell cultures used in this investigation to accumulate vanillic acid glycoside (3.1.1) which may be a product of the hydrolysis of vanilloyl-SCoA followed by glycosylation. For vanillin to be produced, vanilloyl-SCoA would have to be reduced and here again there are several possibilities leading to the formation of vanillylamine as described in 4.4.

Future Work

This research begins to answer some of the questions about the biosynthetic relationships between various phenolic compounds and capsaicin, but there are many interesting questions which still remain unanswered. Further identification of the limiting steps in capsaicin synthesis, followed by characterization of the rate limiting enzyme needs to be carried out. Three major topics which could be studied in the future are: 1. the biosynthetic pathway of hydroxycinnamoyl glycosides in fruits, 2. determination of the limiting step in capsaicin biosynthesis and 3. strategies to increase capsaicin production in cell cultures.

1. Biosynthetic pathway of hydroxycinnamoyl glycosides

The following work is required to prove the proposed pathway presented in this thesis (4.1.2 and 4.1.3):

- a. Measurement of p-coumaroyl-SCoA ligase activity in the whole fruit and in individual tissue compartments in developing fruits, especially before and during the onset of capsaicin synthesis to establish the involvement of p-coumaroyl-SCoA in the synthesis of hydroxycinnamoyl glycosides.
- b. Pulse chase labelling in fruits before the onset of capsaicin to observe the interconversion between the hydroxycinnamoyl glycosides as proposed in Fig. 4.2.
- c. Isolation and elucidation of the structure of the three hydroxycinnamoyl glycosides to enable a study to be made of the enzymes involved in their interconversion.
- d. Study of the enzymes which catalyze the formation of caffeoyl and 3,4-dimethoxycinnamoyl glycosides to determine whether these compounds are synthesized from the corresponding free acids and sugar or from p-coumaroyl glycoside.

2. Limiting step(s) in capsaicin biosynthesis

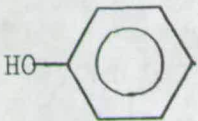
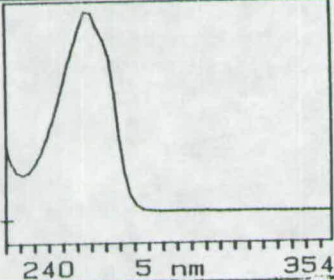
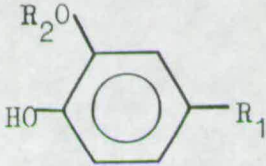
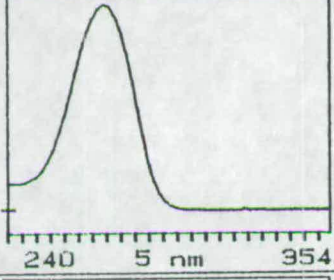
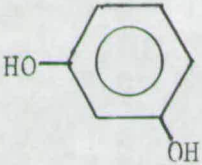
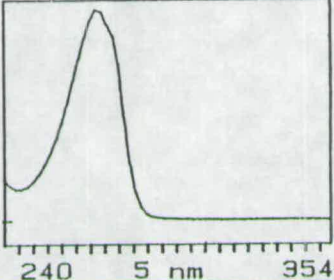
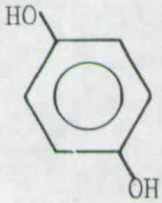
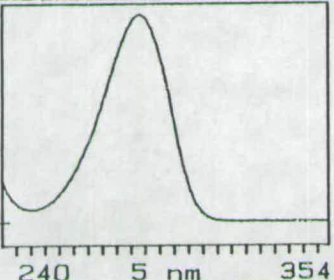
It has been suggested that the rate limiting step in capsaicin biosynthesis is one of the reactions in the aromatic pathway after β oxidation of hydroxycinnamic acid. Unfortunately, most of the radiolabelled precursors required to establish the pathway after β -oxidation of the hydroxycinnamic acids leading to vanillylamine are not available commercially, but evidence for their involvement may be obtained by studying the enzymes which catalyse the reduction of hydroxybenzoates and transamination of hydroxybenzaldehyde as presented in Fig. 4.7. In addition, use of the tracers [MeO- ^{14}C] ferulic and [MeO- ^{14}C] vanillin already synthesized by Tomimura & Terashima (1979) and Funk & Brodelius (1990a) would help to establish the pathway of capsaicin biosynthesis.

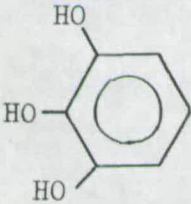
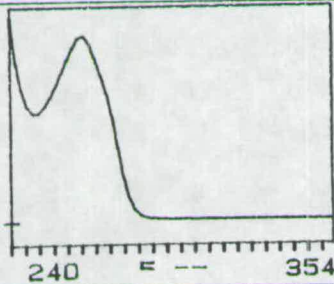
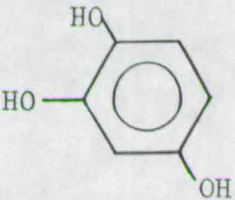

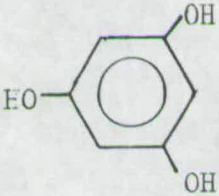
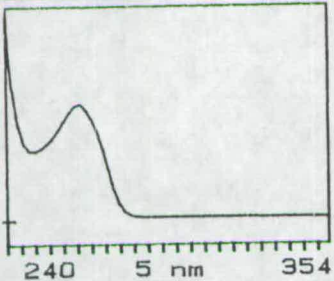
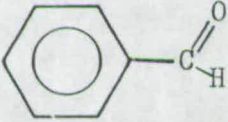
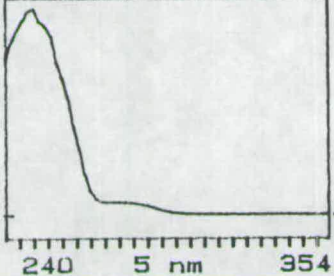
3. Strategy to increase capsaicin production in cell cultures

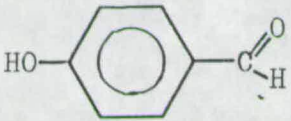
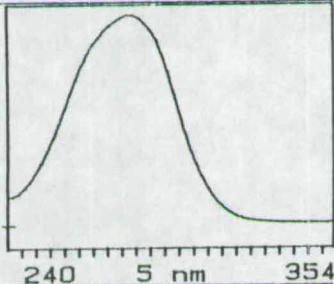
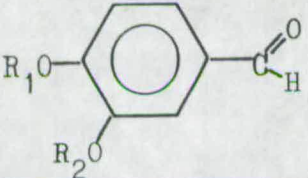
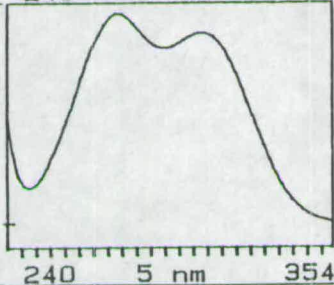
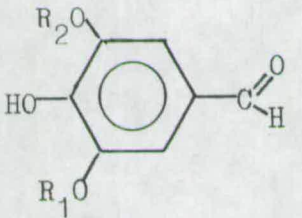
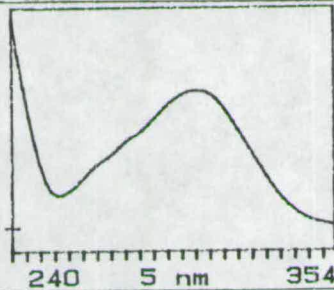
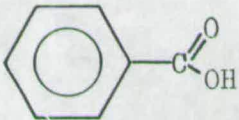
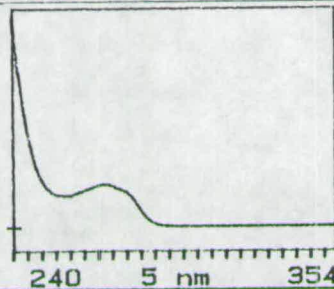
Despite various attempts to increase capsaicin production in cell cultures, the capsaicin yield is variable and low. It may be that the biochemical capacity of cell cultures is similar to fruits before the onset of capsaicin synthesis or to other tissues which do not synthesize capsaicin. Identification of the reasons for low yields in cell cultures including the limiting step of capsaicin biosynthesis could be followed by genetic manipulation to activate the expression of the gene which codes for the enzyme concerned. Following successful activation of the limiting enzyme, further treatments such as increasing PAL activity by elicitation (Holden *et al.*, 1988a,b), inhibition of lignin synthesis (Funk and Brodelius, 1990a), manipulation of the culture environment (Lindsey and Yeoman, 1984a; Lindsey, 1985, 1986a,b) and using PFP (p-fluorophenylalanine) resistant cell lines (Salgado-Garciglia and Ochoa-Alejo, 1990) could result in consistently enhanced capsaicin production by cell cultures.

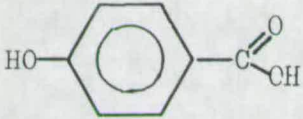
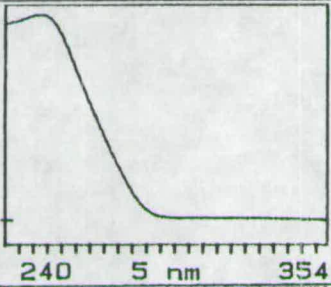
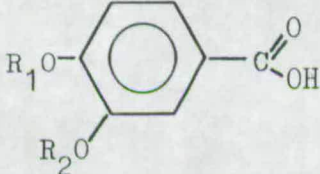
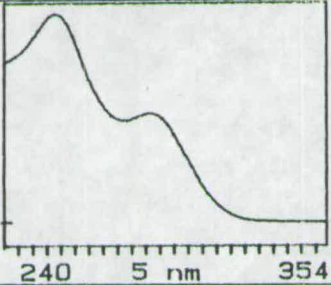
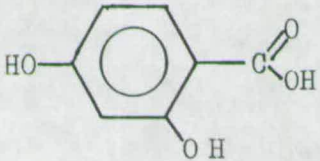
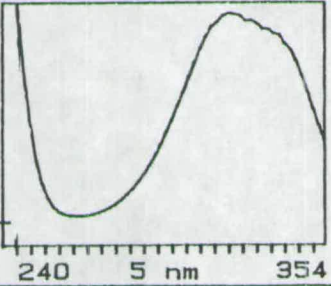
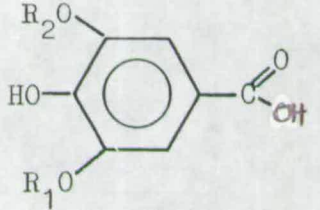
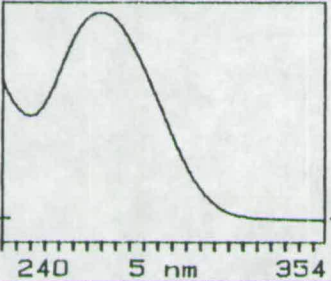
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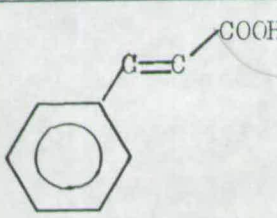
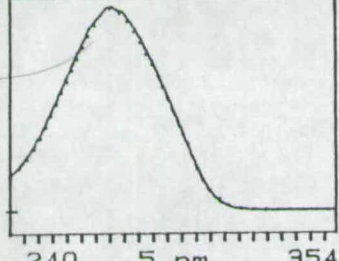
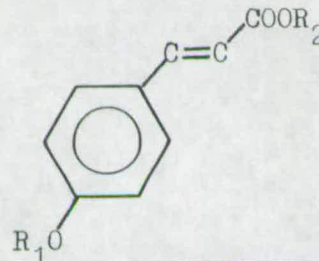
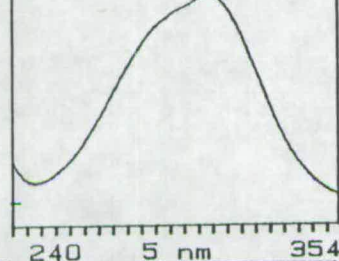
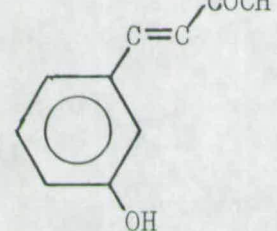
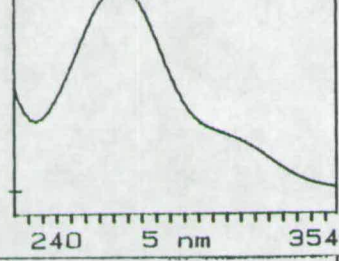
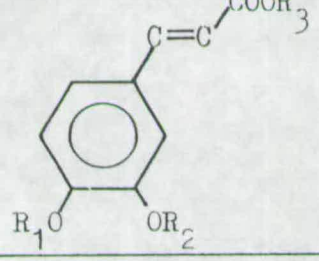
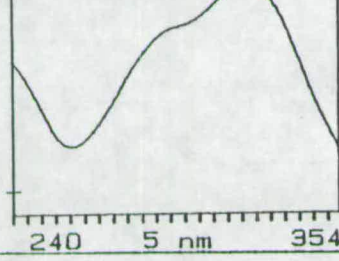
UV spectra of various ^{C₆,}C₆-C₁ and C₆-C₃ compounds eluted from a Hewlett Packard HP1090 liquid chromatograph in conjunction with HP1040 diode array detector fitted with 4.5μl flow cell.

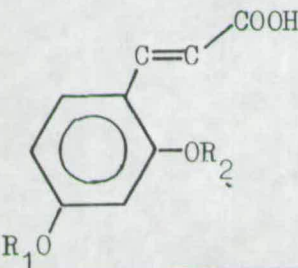
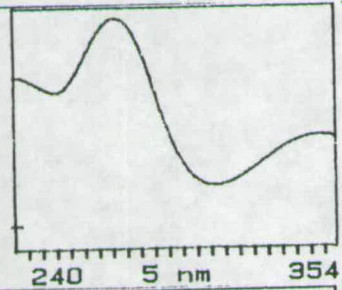
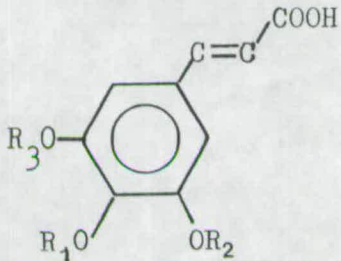
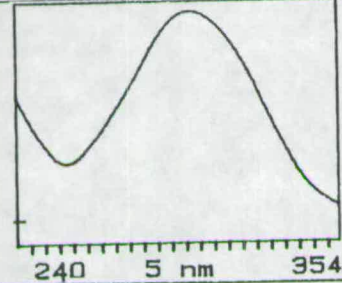
Structure	UV Spectrum	Compound	R ₁	R ₂	R ₃	λ _{max} (nm)
		Phenol	-	-	-	268
		Catechol Capsaicin Vanillylamine Vanillylalcohol	H C ₁₁ H ₂₀ NO CH ₂ NH ₂ CH ₂ OH	H CH ₃ CH ₃ CH ₃	- - - -	274 278 278 278
		Resorcinol	-	-	-	272
		Hydroquinone	-	-	-	288

Structure	UV Spectrum	Compound	R ₁	R ₂	R ₃	λ _{max} (nm)
		Pyrogallol	-	-	-	266
		Hydroquinol	-	-	-	-
		Phloroglucinol	-	-	-	266
		Benzaldehyde	-	-	-	248

Structure	UV Spectrum	Compound	R ₁	R ₂	R ₃	λ _{max} (nm)
		p-OH-benzaldehyde	-	-	-	282
		Protocatechuic aldehyde	H	H	-	278, 309
		Vanillin	H	CH ₃	-	280, 308
		Iso-Vanillin	CH ₃	H	-	280, 308
		3,4,5-Trihydroxybenzaldehyde	H	H	-	300
		Syringaldehyde	CH ₃	CH ₃	-	300
		Benzoic acid	-	-	-	271

Structure	UV Spectrum	Compound	R ₁	R ₂	R ₃	λ _{max} (nm)
		p-Hydroxybenzoic acid	-	-	-	254
		Protocatechuic acid Vanillic acid Iso-vanillic acid	H H CH ₃	H CH ₃ H	- - -	253, 291 260, 290 260, 284
		Gentisic acid	-	-	-	320
		Gallic acid Syringic acid	H CH ₃	H CH ₃	- -	272 274

Structure	UV Spectrum	Compound	R ₁	R ₂	R ₃	λ _{max} (nm)
		Cinnamic acid	-	-	-	276
		p-Coumaric acid	H	H	-	309
		p-Methylcoumaric acid	CH ₃	H	-	309
		p-Coumaroylglucose	H	Glucose	-	314
		m-Coumaric acid	-	-	-	278
		Caffeic acid	H	H	H	322
		Chlorogenic acid	H	H	Quinate	319
		Ferulic acid	H	CH ₃	H	322
		Iso-ferulic acid	CH ₃	H	H	322
		3,4-OCH ₃ -Cinnamic acid	CH ₃	CH ₃	-	320

Structure	UV Spectrum	Compound	R ₁	R ₂	R ₃	λ _{max} (nm)
		2,5-OCH ₃ -Cinnamic acid	CH ₃	CH ₃	-	276, 350
		Sinapic acid 3,4,5-OCH ₃ -Cinnamic acid	H CH ₃	CH ₃ CH ₃	CH ₃ CH ₃	324 302

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